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13. ABSTRACT (Maximum 200 Words)

Androgen Receptor Trapped clone-27 (ART-27) is a transcriptional coactivator of the androgen receptor (AR) that was identified in yeast two-hybrid screen using the AR as bait (see Markus et al). Recent studies suggest that the ART-27 protein is expressed in normal differentiated epithelial cells of the prostate and breast, in contrast to the stroma, where ART-27 is not expressed. Expression of ART-27 in LNCaP cells, an androgen-dependent prostate cancer cell line, reduces androgen-mediated cellular proliferation, suggesting that ART-27 plays a role in suppressing cell growth. Consistent with a growth suppressive function, ART-27 protein is not detected in human prostate cancers or in the undifferentiated developing fetal prostate gland, whereas at a later stage of fetal prostate development when differentiated epithelial cells are evident ART-27 is detected. These results suggest that the lack of ART-27 in prostate cancer may occur as a result of de-differentiation. We propose that ART-27, via its activity as an AR coactivator, controls AR responsive genes that suppress proliferation and promote differentiation. Strategies to restore ART-27 expression could effectively treat local or metastatic prostate cancers by reestablishing a differentiated state and inhibiting cellular proliferation.

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INTRODUCTION

Activation of androgen receptor (AR) plays a critical role in the development and progression of prostate cancer. The AR transduces the signaling information conveyed by androgens and controls gene expression through binding with critical transcriptional coregulatory proteins termed coactivator. This in turn, allows the AR to "switch on" or "switch off" genes important for prostate cancer growth. Therefore, identification and characterization of the AR coregualtory molecules will provide important new targets for the treatment of these diseases.

BODY

Statement of Work (Year 1).

Task1. Identification and Characterization of AR AF-1 Interacting Proteins

a. Cloning and characterization of the AR AF-1 Interacting Protein, ART-27
b. Expression analysis of ART-27
b. Expression of ART-27
c. Specificity of ART-27 interaction

This task has been completed and is reported in:

Markus, S.M., et al., (2002) Identification and characterization of ART-27, a novel coactivator for the androgen receptor N-terminus, *Mole. Biol. Cell* 13, 670-682.

Task 2: Role of AR AF-1 Interacting Proteins in prostate cancer (years 2 and 3)

This task has been largely completed and reported in:

Taneja, S.S., et al. Androgen receptor coactivator ART-27, in androgen-mediated cell growth, cancer and development *(submitted)*

Description of Project completed in the past year (Task 2)

We have, through a unique screening process, identified several proteins that interact with the AR N-terminus. The focus of our studies has been on a molecule, termed androgen receptor trapped clone 27 (ART-27), which binds to the AF-1 region in the N-terminus. Our previously published work has shown that ART-27 increases AR-dependent transactivation (see Markus *et al. Mole. Biol. Cell* 13, 670-682). More recently, we have developed antibodies to ART-27 and investigated its expression in normal and malignant prostate cancer tissues by immunohistochemistry.

ART-27 protein is expressed in normal differentiated epithelial cells of the prostate and breast, in contrast to the stroma, where ART-27 is not expressed. Expression of ART-27 in LNCaP cells, an androgen-dependent prostate cancer cell line, reduces androgen-mediated cellular proliferation, suggesting that ART-27 plays a role in slowing cell growth. Consistent with a growth suppressive function, ART-27 protein is not detected in human prostate cancers or in the undifferentiated developing fetal prostate gland, whereas at a later stage of fetal prostate development when differentiated epithelial cells are evident ART-27 is detected. These results suggest that the lack of ART-27 in prostate cancer may occur as a result of de-differentiation. We propose that ART-27, via its activity as an AR coactivator, controls AR responsive genes that suppress proliferation and promote differentiation. Thus, ART-27 is the first AR cofactor shown to be subject to both cell type and developmental regulation in humans. These findings are described in the manuscript enclosed.

Future directions:

To definitively determine the role of ART-27 in prostate differentiation and growth control, we propose to use mice as a model system in which to modulate the level of ART-27. Mice lacking ART-27 will be created by targeted mutagenesis and in an opposing strategy, transgenic mice that over-express ART-27 will be made. ART-27 will be over-expressed under the regulation of the rat probasin promoter, which has been shown to direct prostate epithelial cell-specific expression in mice. We anticipate in the mice lacking ART-27 that prostate epithelial cells would continue to proliferate and fail to differentiate, whereas cells that overexpress ART-27 would have the opposite phenotype. Strategies to restore ART-27 expression could effectively treat local or metastatic prostate cancers by reestablishing a differentiated state and inhibiting cellular proliferation.

KEY RESEARCH ACCOMPLISHMENTS:

- Identified ART-27 as a protein that interacts with the AR N-terminal subdomain spanning amino acids 153-336, including AF-1a (154-167) and a part of AF-1b (295-459)

- ART-27 enhances AR transcriptional activation when overexpressed in mammalian cells.

- Thus, ART-27 represents a novel AR N-terminus-associated coactivator.

- ART-27 protein is expressed in normal differentiated epithelial cells of the prostate, but not the stroma.

- Expression of ART-27 in LNCaP cells reduces androgen-mediated cellular proliferation, suggesting that ART-27 plays a role in suppressing cell growth.

- ART-27 protein is not detected in human prostate cancers or in the undifferentiated developing fetal prostate gland, whereas at a later stage of fetal prostate development when differentiated epithelial cells are evident ART-27 is detected.

REPORTABLE OUTCOMES:

1) Markus, S.M., Taneja, S.S.,Logan, S.K., Li, W., Ha, S., Hittelman, A.B., Rogatsky, I., and Garabedian, M.J., (2002) Identification and characterization of ART-27, a novel coactivator for the androgen receptor N-terminus, *Mole. Biol. Cell* 13, 670-682

2) Taneja, S.S., Ha, S., Swenson, N., Rome, S., Walden, P., Huang,H-Y., Shapiro, E., Garabedian, M.J. and Logan, S., Androgen receptor coactivator ART-27, in androgen-mediated cell growth, cancer and development *(submitted)z*

CONCLUSIONS:

We have identified and characterized a novel AR cofactor, termed ART-27. We propose that ART-27, via its activity as an AR coactivator, controls AR responsive genes that suppress proliferation and promote differentiation. Strategies to restore ART-27 expression could effectively treat local or metastatic prostate cancers by reestablishing a differentiated state and inhibiting cellular proliferation.

APPENDICIES:

Markus S.M. et al, *Mole. Biol. Cell* 13, 670-682
Taneja, S.S., et al, *submitted*

List of Personnel receiving pay from the research effort:

Steven M. Markus
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Identification and Characterization of ART-27, a Novel Coactivator for the Androgen Receptor N Terminus

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The androgen receptor (AR) is a ligand-regulated transcription factor that stimulates cell growth and differentiation in androgen-responsive tissues. The AR N terminus contains two activation functions (AF-1a and AF-1b) that are necessary for maximal transcriptional enhancement by the receptor; however, the mechanisms and components regulating AR transcriptional activation are not fully understood. We sought to identify novel factors that interact with the AR N terminus from an androgen-stimulated human prostate cancer cell library using a yeast two-hybrid approach designed to identify proteins that interact with transcriptional activation domains. A 157-amino acid protein termed ART-27 was cloned and shown to interact predominantly with the $AR_{153–336}$, containing AF-1a and a part of AF-1b, localize to the nucleus and increase the transcriptional activity of AR when overexpressed in cultured mammalian cells. ART-27 also enhanced the transcriptional activation by $AR_{153–336}$ fused to the LexA DNA-binding domain but not other AR N-terminal subdomains, suggesting that ART-27 exerts its effect via an interaction with a defined region of the AR N terminus. ART-27 interacts with AR in nuclear extracts from LNCaP cells in a ligand-independent manner. Interestingly, velocity gradient sedimentation of HeLa nuclear extracts suggests that native ART-27 is part of a multiprotein complex. ART-27 is expressed in a variety of human tissues, including sites of androgen action such as prostate and skeletal muscle, and is conserved throughout evolution. Thus, ART-27 is a novel cofactor that interacts with the AR N terminus and plays a role in facilitating receptor-induced transcriptional activation.

INTRODUCTION

The androgen receptor (AR) is a member of the steroid receptor (SR) family of transcriptional regulatory proteins that transduces the signaling information conveyed by androgens (Wilson *et al.*, 1991; Chang *et al.*, 1995). On androgen binding, the AR is released from the repressive effects of an Hsp90-based regulatory complex, allowing the receptor to either activate or inhibit transcription of target genes (Picard *et al.*, 1990; Jenster *et al.*, 1991, 1992; Duina *et al.*, 1996; Fang *et al.*, 1996, 1998; Segnitz and Gehring, 1997). In addition to its role in male sex determination, AR mediates normal prostate development as well as malignant growth by regulating genes and signaling pathways involved in cellular proliferation (Brinkmann *et al.*, 1992; Hakimi *et al.*, 1996; Trapman and Brinkmann, 1996; Dorkin and Neal, 1997; Dorkin *et al.*, 1997; Jenster, 1999).

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The mechanisms underlying the specificity of AR regulation of gene expression remain enigmatic. The DNA-binding domain of AR is highly conserved among SRs and recognizes the same hormone response element (HRE) as does the glucocorticoid receptor (GR). Although subtle preferences for particular HREs may contribute to the specificity of AR-mediated transcriptional response (Nelson *et al.*, 1999; Schoenmakers *et al.*, 2000), recent evidence suggests that the AR cell- and promoter-specific regulation is generated through interactions with regulatory proteins termed coactivators and corepressors (Cleutjens *et al.*, 1997; Scheller *et al.*, 1998). For instance, agonist binding to the AR C-terminal activation function-2 (AF-2) promotes a conformational change and the formation of a surface for protein-protein contacts between AF-2 and additional transcriptional regulatory factors, which, in turn, modulate the transcriptional activity of target genes (Onate *et al.*, 1995; Smith *et al.*, 1996; Voegel *et al.*, 1996; Yeh and Chang, 1996; Chen *et al.*, 1997; Hong *et al.*, 1997; Li *et al.*, 1997; Torchia *et al.*, 1997; Kang *et al.*, 1999). Because the growing number of SR coactivators and corepressors appear to function widely across the SR

family with conserved AF-2 regions (Glass and Rosenfeld, 2000), it is unlikely that these factors alone determine specificity of receptor transcriptional regulation. In contrast, the N-terminal transcriptional regulatory regions, which are diverse throughout the SR family, may represent an important determinant of SR specificity, conceivably through the recruitment of distinct coregulators. Indeed, Hittelman *et al.* (1999) recently identified DRIP150 as a GR N-terminal coactivator that does not interact with the N termini of other SRs, including AR. However, the mechanisms of transcriptional activation by the AR N terminus are not well understood, and although the list of proteins proposed to bind to the AR N terminus is expanding (Gelman *et al.*, 1999; Hsiao and Chang, 1999; Ma *et al.*, 1999; Lee *et al.*, 2000), it is likely that additional AR-binding partners remain to be identified.

Regions of the AR N terminus important for transcriptional activation have been identified by expressing and analyzing receptor deletion derivatives or fusion proteins in mammalian cells and in cell-free systems. At least two distinct activation domains within the AR N terminus have been identified, AF-1a (residues 154–167) and AF-1b (residues 295–459), both of which are required for full transcriptional activation mediated by the receptor (Chamberlain *et al.*, 1996). The AR N-terminal residues 142–485 have also been shown to activate a minimal promoter construct in a cell-free transcription system and to selectively interact with the transcription factors TFIIF and the TATA-binding protein (TBP), suggesting a direct contact with the general transcription factors (McEwan and Gustafsson, 1997). Protein-protein interaction studies have recently suggested contacts between the AR N terminus and the TATA-element modulating factor, or ARA160, which increases AR transcriptional activity when overexpressed in certain cell types (Hsiao and Chang, 1999).

Interestingly, the growth of both normal and tumoregenic prostate cells is regulated by AR. A number of prostate cell lines display elevated AR-dependent transcriptional activation relative to nonprostatic cell lines; the AR N terminus appears responsible for this enhanced receptor activity (Gordon *et al.*, 1995), suggesting the existence of cofactors that modulate transcriptional activation by the AR N-terminal activation domain in prostate epithelial cells. Together, the current data support the notion that the AR N terminus contains multiple surfaces capable of interaction with general transcription factors and possibly additional adapter proteins. Recently, a patient with androgen insensitivity syndrome was described whose cells lack AR transcriptional activity, probably through the loss of an as yet unidentified AR N-terminal cofactor (Adachi *et al.*, 2000), underscoring the importance of the AR N terminus and associated factors in human disease.

In a yeast two-hybrid screen designed to identify factors that interact with transcriptional activation domains, we isolated from an androgen-stimulated prostate cancer cell library a novel factor that associates with the AR N terminus. We examined the specificity and molecular determinants of this interaction and characterized the effects on SR-dependent transcriptional activation in mammalian cells.

MATERIALS AND METHODS

Construction of Plasmids

Yeast expression vectors for the LexA-AR fusion protein, LexA-AR_{18-500}, was created by digesting the rat AR N terminus with *Eco*RI-*Xho*I and subcloned into the pEG202 vector that was digested with *Eco*RI-*Xho*I. The subregions of the rat AR N terminus (LexA-AR_{18-156}, LexA-$AR_{153-336}$, and LexA-$AR_{336-500}$) were generated as follows: for LexA-AR_{18-156}, pEG202:AR_{18-500} was digested with *Eco*RI-*Pvu*II and the insert was ligated into pEG202, which was digested with *Not*I, the 5′ overhang filled in with Klenow fragment, and *Eco*RI; for LexA-$AR_{153-336}$, pEG202:AR_{18-500} was digested with *Bst*YI-*Afl*II, filled in with Klenow, and ligated into pEG202, which was digested with *Bam*HI-*Xho*I with ends filled in; for LexA-$AR_{336-500}$, pEG202:AR_{18-500} was digested with *Bst*YI-*Xho*I and the insert was ligated into pEG202, which was digested with *Bam*HI-*Xho*I. For mammalian expression, the LexA DNA-binding domain AR N-terminal fusions were excised with *Hin*dIII-*Xho*I, and the insert was ligated into pcDNA3. Yeast two-hybrid "bait" proteins, B42-AR_{18-156}, B42-$AR_{153-336}$, B42-$AR_{336-500}$, and B42-AR_{18-500} were constructed by subcloning respective *Eco*RI-*Xho*I fragments from pEG202 into the corresponding sites in pJG4-5. The LexA-LNCaP cell cDNA library was purchased from Origene Technologies (Rockville, MD). The $AR_{579-901}$ was PCR amplified using the following primers: (forward with a *Bgl*II site) 5′-AGATCTTAAGCAGAAATGATTGCACCATTG-3′ and (reverse with an *Xho*I site) 5′-GTAGATAAAGGTGTGTGTCACTGAGCTC-3′, ligated into pGEM:T-easy (Promega, Madison, WI), excised with *Bgl*II-*Xho*I, and the insert was ligated into pEG202, which was digested with *Bam*HI-*Xho*I. pEG202:$AR_{579-901}$ was then digested with *Eco*RI-*Xho*I and the insert was ligated into pJG4-5.

The LexA–ART-27 (androgen receptor trapped clone 27) C-terminal truncations 1–45, 1–67, and 1–127 were constructed by digesting pEG202:ART-27 with *Pvu*II, *Bsp*MI, and *Sty*I, respectively, filling in their 5′-overhangs, digesting with *Mlu*I, and ligating the inserts into pEG202, which was digested with *Not*I/Klenow-*Mlu*I. The LexA–ART-27 N-terminal truncations 46–157, 68–157, and 127–157 were constructed as follows: for LexA–ART-27_{46-157}, pEG202:ART-27 was digested with *Pvu*II-*Xho*I and ligated into pEG202, which was digested with *Bam*HI/Klenow and *Xho*I; for LexA–ART-27_{68-157}, pEG202:ART-27 was digested with *Bsp*MI/Klenow and *Xho*I, and the insert was ligated into pEG202, which was digested with *Bam*HI/Klenow and *Xho*I; for LexA–ART-$27_{127-157}$, pEG202:ART-27 was digested with *Sty*I/Klenow and *Xba*I, and the insert was ligated into pEG202, which was digested with *Eco*RI/Klenow and *Xba*I. For LexA–ART-$27_{1-45/127-157}$, PCR primers were designed as follows: ART-27_{1-45} (forward pEG202 primer) 5′-TTGGGGTTATTCGCAACGG-3′, (reverse with *Bam*HI site) 5′-GAACTGGATCCCTGCTCATATACCTTGTCTCGATG-3′; ART-$27_{127-157}$ (forward with *Bam*HI site) 5′-GAACTGGATCCACCAAGGACTCCATG-3′, (reverse pEG202 primer) 5′-CGGAATTAGCTTGGCTGC-3′. The two separate fragments were PCR amplified, and the resulting products were digested as follows: ART-27_{1-45} with *Eco*RI-*Bam*HI, ART-$27_{127-157}$ with *Bam*HI-*Xho*I, and the two inserts were ligated together into pEG202, which was digested with *Eco*RI-*Xho*I. For mammalian expression, the pEG202:ART-27 was digested with *Eco*RI-*Xho*I and subcloned into a pcDNA3 vector that has an N-terminal hemagglutinin (HA) epitope (pcDNA3-HA).

pJG4–5:Sp1A_{83-262}, pJG4–5:Sp1$B_{263-542}$, pJG4–5:TAF130$_{270-700}$, and pJG4–5:CREB$_{3-296}$ were provided by N. Tanese (New York University School of Medicine, New York). pJG4–5:SRC-$1_{374-800}$ was provided by H. Samuels (New York University School of Medicine). pJG4–5:$GR_{107-237}$, pJG4–5:$GR_{107-237}$ 30IIB, and pJG4–5:VP16 were previously described (Hittelman *et al.*, 1999). The pJK103 reporter plasmid, which contains a single LexA operator linked to β-galactosidase, was used in activity assays of the LexA fusion proteins and in the modified two-hybrid assay. The pΔ4X-LALO-luciferase reporter plasmid, which contains four LexA operators upstream of a minimal *Drosophila* alcohol dehydrogenase promoter linked to luciferase, was used in mammalian activity assays to

monitor the intrinsic transcriptional activity of the LexA fusion proteins. The pcDNA3:hAR expression plasmid was used to produce full-length human AR, pMMTV:luciferase reporter was used to assay AR transcriptional activity, and pCMV:LacZ constitutively expressed β-galactosidase, a marker for transfection efficiency. Other receptor expression plasmids include pcDNA3-human estrogen receptor (ER)-α, pCMV5-human ERβ (Su *et al.*, 2001), and pRep4-human thyroid hormone receptor β-1 (TRβ-1). The ER (XETL) and TR (pGL3-DR4) reporter plasmids have been previously described (Sharif and Privalsky, 1992; Su *et al.*, 2001).

Modified Yeast Two-Hybrid System

To identify proteins that interact with AR N terminus, we used a modification of the yeast two-hybrid system that allows for the selection of proteins that interact with transcriptional activators (Hittelman *et al.*, 1999). Linking amino acids 18–500 of the rat AR to the B42 activation domain created the AR "bait" (pJG4–5:AR_{18-500}). The "prey" is created by fusing an androgen-stimulated LNCaP cell cDNA library to the LexA DNA-binding domain (pEG202:LNCaP cell cDNA library) rather than to an activation domain. The yeast strain EGY188 was transformed by the lithium acetate method with 1) pJG4–5:AR_{18-500}, 2) pEG202:LNCaP cell cDNA library, and 3) pJK103, a β-galactosidase reporter gene with a single LexA operator. Potential interacting proteins were selected by plating the cDNA library expressing transformants onto leu^-/X-gal^+ galactose plates. Library proteins that interact with AR stimulate expression of Lex-responsive *Leu2* and β-galactosidase reporter genes. To eliminate library plasmids that may possess intrinsic activation potential, $Leu2^+$/$LacZ^+$ colonies were replica plated onto glucose plates, conditions in which the AR protein is not expressed. Colonies that activated β-galactosidase expression on galactose plates, when AR is expressed, but not glucose plates, were deemed true interactors and further analyzed.

Quantitative Liquid β-Galactosidase Assay

Yeasts were grown in selective liquid media containing 2% glucose for ~12 h, pelleted, washed once with sterile H_2O, normalized to cell number, and resuspended to an optical density (OD_{600}) of 0.15 in 2% galactose/1% raffinose. β-Galactosidase assays were performed 12 h later as described previously (Garabedian and Yamamoto, 1992).

Mammalian Cell Culture and Transient Transfection Assays

A human cervical carcinoma cell line (HeLa), a human prostate cancer cell line (PC-3), and SV40 T-antigen expressing monkey kidney cells (COS-1) were obtained from the American Type Culture Collection and maintained in DMEM (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT), 50 U/ml each of penicillin and streptomycin (Invitrogen), and 2 mM L-glutamine (Invitrogen). The androgen-dependent prostate cancer cell line (LNCaP) was maintained in RPMI-1640 (Invitrogen) supplemented with 10% FBS, 50 U/ml each penicillin and streptomycin, and 2 mM L-glutamine. For transfections, HeLa cells were seeded in 35-mm dishes at a density of 1.3×10^5, washed once with serum-free medium, and transfected with 0.2 μg of pcDNA3:human AR, 0.1 μg of pMMTV-Luc, 0.05 μg of pCMV-LacZ, and the indicated amounts of pcDNA3:HA–ART-27 using 5 μl of Lipofectamine reagent (Invitrogen) in a total volume of 1 ml of serum-free, phenol red-free DMEM per 35-mm dish according to the manufacturer's instructions. Approximately 4 h posttransfection, the transfection mixture was removed, and the cells were refed with 2 ml of DMEM-10% FBS, allowed to recover for 3–5 h, and fed again with fresh DMEM-10% FBS supplemented with 100 nM R1881, dexamethasone, 17β-estradiol, triac, or an identical volume of 100% ethanol and incubated for 12 h. Transfected cells were washed once in phosphate-buffered saline (PBS) and harvested in 1X reporter lysis buffer (Promega) according to the manufacturer's instructions. PC-3 cells were seeded in 35-mm dishes at a density of 1.1×10^5 and transfected as above. To assay the LexA-AR N terminus, 0.5 μg of pcDNA3-LexA:AR derivatives, 1.0 μg of pcDNA3-HA:ART-27 or empty vector, 1.0 μg of pΔ4X-LALO reporter, and 0.25 μg of pCMV-LacZ were transfected into HeLa cells that were transfected as above using 6 μl of Lipofectamine per 35-mm dish. Luciferase activity was quantitated in a reaction mixture containing 25 mM glycylglycine, pH 7.8, 15 mM $MgSO_4$, 1 mM ATP, 0.1 mg/ml bovine serum albumin (BSA), 1 mM dithiothreitol using a Lumen LB 9507 luminometer (EG&G Wallac, Gaithersburg, MD), and 1 mM D-luciferin (PharMingen, San Diego, CA) as substrate.

Preparation of the ART-27 Antibody

A 16-amino acid peptide that corresponds to the ART-27 C-terminal amino acids 142–157 (R-E-L-Q-G-L-Q-N-P-G-K-P-H-H) with an additional cysteine residue at the N terminus was synthesized by Anaspec (San Jose, CA), coupled to KLH, and used to immunize rabbits by Covance Research Products (Denver, PA). The IgG fraction was purified from the serum by protein A chromatography, and the ART-27 antibody was obtained by affinity purification using the ART-27 peptide immunogen coupled to Affi-gel 15 (Bio-Rad Laboratories, Hercules, CA) resin.

Northern Blotting

Cells were cultured in 100-mm dishes for the indicated periods of time with appropriate treatments (see figure legends), the media were aspirated, and the cells were lysed by adding 3 ml/dish of RNA STAT-60 reagent (Tel-Test, Friendswood, TX). Total RNA was isolated from cell homogenates according to the manufacturer's instructions, denatured at 65°C for 15 min, chilled on ice, and separated on a 1.2% agarose/6% formaldehyde denaturing gel (10 μg RNA/lane). Equivalent loading was verified by ethidium bromide staining of rRNA. RNA was transferred to Duralon paper (Stratagene, San Diego, CA), UV cross-linked to the membrane, and hybridized to a cDNA probe using QuikHyb hybridization mixture (Stratagene) as described by the manufacturer. A 0.9-kb cDNA fragment encoding ART-27 was labeled with [α-^{32}P]dCTP (NEN, Boston, MA) using RediPrime random priming labeling kit (Amersham Pharmacia Biotech, Piscataway, NJ) using the manufacturer's instructions. Blots were washed and exposed to BioMax film (Kodak, Rochester, NY) at −80°C for autoradiography. Hybridization of ART-27 to a multiple tissue Northern blot membrane (CLONTECH, Palo Alto, CA) was performed according to the manufacturer's instructions.

Coimmunoprecipitation

Full-length AR and HA–ART-27 were translated in vitro using TNT Quick Coupled Transcription/Translation System (Promega) in the presence of [^{35}S]methionine. The radiolabeled proteins were incubated as indicated in binding buffer (20 mM Tris, pH 7.9, 170 mM KCl, 20% glycerol, 0.2 mM EDTA, 0.05% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 4 mg/ml BSA) for 1 h at 4°C. α-HA (12CA5, 1 μg) antibody (Boehringer Mannheim, Indianapolis, IN) was incubated with the radiolabeled proteins for 1 h at 4°C. Protein A Sepharose Fast Flow beads (30 μl, Amersham Pharmacia Biotech) were incubated with the respective reaction mixtures for 1 h at 4°C. The beads were washed three times in lysis buffer, resuspended in 2× SDS sample buffer, and boiled for 3 min; the associated proteins were resolved by SDS-PAGE and visualized by autoradiography.

LNCaP-stable cell lines were generated that express ART-27 with a C-terminal FLAG epitope in an inducible manner (LNCaP-Tet-on-ART-27-FLAG cells). This line was created in two steps. First, LNCaP cells were transfected with the pTet-On vector using

DOTAP (Roche, Summerville, NJ), and resistant colonies were selected at 500 μg/ml Geneticin (Invitrogen). Clones were transferred to a 24-well dish coated with fibronectin (10 μg/ml; Invitrogen), expanded, and screened for Tet-dependent activation by measuring pRevTRE-luciferase reporter gene activity in the absence and presence of 1 μg/ml doxycyclin (Sigma). A LNCaP clone displaying tight Tet-dependent regulation was transfected with pRevTRE: ART-27 (C-FLAG) as above, and resistant colonies were selected at 150 μg/ml hygromycin B (Invitrogen). Multiple clones were screened for Tet-dependent activation by immunoblotting for the FLAG epitope resident on ART-27 in the absence and presence of 1 μg/ml doxycyclin. Small-scale nuclear extracts were prepared as described by Lee *et al.* (1988) from two confluent 100-mm dishes of LNCaP-Tet-on-ART-27-FLAG cells that had been induced overnight with 1 μg/ml doxycyclin and either 10 nM R1881 or an equivalent volume of ethanol vehicle. The total protein concentration in the nuclear extracts was equalized with lysis buffer, and 10 μl of either preimmune or immune ART-27 antisera were added. After incubation at 4°C for 1.5 h, 40 μl of Protein A Sepharose Fast Flow beads (Amersham Pharmacia Biotech) were added and incubated for 1 h at 4°C. The immune complexes were washed three times in wash buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 10% glycerol, 1% Triton X-100), resuspended in 2× SDS sample buffer, and boiled for 3 min; the associated proteins were resolved by SDS-PAGE, transferred to Immobilon paper (Millipore, Bedford, MA), and probed with either an AR polyclonal antibody (sc-816; Santa Cruz Biochemicals, Santa Cruz, CA) or an affinity-purified ART-27 antibody.

Immunoblotting

Yeast protein extracts were prepared from 2-ml cultures and lysed using glass beads as previously described (Knoblauch and Garabedian, 1999). Lysates from mammalian cells were prepared as described by Hittelman *et al.* (1999). For immunoblotting, HeLa cell nuclear extracts, which were untreated, phorbol 12-myrisate 13 acetate (TPA; 50 ng/ml) for 2 h before harvesting, or serum stimulated (cells cultured for 24 h in medium containing 0.5% serum and serum stimulated [20%] for 2 h before harvesting), and PC-3 cell nuclear extracts were purchased from Geneka Biotechnology (Montreal, Quebec, Canada). Large-scale HeLa cell nuclear extracts for the velocity gradient sedimentation analysis were prepared from the nuclei of 5 liters of HeLa cells (Cellex Biosciences, Minneapolis, MN) by the method of Dignam and Roeder (Dignam *et al.*, 1983). Protein concentration in extracts was normalized by the Bradford assay (Bio-Rad), separated on SDS/4–20% polyacrylamide gels (Novex, San Diego, CA), and transferred to Immobilon paper. Membranes were probed with a polyclonal antibody against LexA (a gift from E. Golemis), a monoclonal antibody to HA (12CA5; Boehringer Mannheim), a monoclonal antibody to TBP (a gift from N. Tanese) or affinity-purified rabbit anti–ART-27 antibody. The blots were developed using horseradish peroxidase-coupled donkey anti-rabbit or sheep anti-mouse antibodies and enhanced chemiluminescence (ECL; Amersham-Pharmacia). Quantitative analysis of immunoblots was performed using the NIH image software package (version 1.62; National Institutes of Health, Bethesda, MD).

Immunofluorescence

HeLa cells were seeded onto poly-D-lysine–coated coverslips, transfected with pcDNA3-FLAG–ART-27, and, 24 h later, washed five times with PBS and fixed in 4% paraformaldehyde in PBS for 20 min at room temperature (RT). Cells were then permeabilized with 0.2% Triton X-100 in PBS for 20 min and incubated in 100 μl of the FLAG monoclonal antibody (M2) diluted to a concentration of 2 μg/ml in blocking solution (5% BSA/Tris-buffered saline [TBS] for 2 h at RT. Cells were washed five times in 1 ml of 0.1% Triton X-100 in PBS, followed by incubation with goat anti-mouse fluorescein-conjugated secondary antibody (Vector Labs, Burlingame, CA) diluted in blocking solution, for 1 h at RT. Secondary antibody was removed by washing the cells five times in 0.1% Triton X-100 in PBS and three times in PBS. Nuclei were stained with 1 μg/ml Hoechst dye H334211 for 10 min, followed by one wash with PBS. Coverslips were mounted onto Citifluor (Ted Pella, Redding, CA), and the fluorescein and Hoechst signals were visualized and photographed using an Axioplan 2 microscope (Zeiss, Thornwood, NY).

Immunohistochemistry

An indirect immunoperoxidase method was used to identify ART-27 in LNCaP and PC-3 tumors grown in nude mice (xenografts). The LNCaP and PC-3 tumor xenografts were removed from the mice and fixed for 2 h in 4% paraformaldehyde in PBS (pH 7.4) at RT. The tissue was dehydrated through ethanol, cleared in chloroform, and embedded in paraffin. Tissue sections (5-μm) were serially cut on a microtome and mounted on slides. Sections were dewaxed in xylene, rehydrated, and washed in TBS, pH 7.4. For antigen retrieval, paraffin sections were heated in a microwave oven for 15 min (900 W, high power) in Target Retrieval Solution (Dako, Carpinteria, CA), cooled, and treated with 3% H_2O_2 for 15 min, rinsed with H_2O, and blocked with 20% normal goat serum for 30 min. Sections were incubated with affinity-purified ART-27 antibody (1:100 dilution) in 10% normal goat serum and washed in TBS; a rabbit secondary biotinylated antibody was added and an avidin-biotin complex formed and developed using diaminobenzidine substrate. Slides were counterstained with hematoxylin.

Velocity Gradient Sedimentation

HeLa cell nuclear extracts (~100 μl of 13 mg/ml) were loaded on top of a 5-ml linear 15–35% (vol/vol) glycerol gradient, with or without 2.4 M urea and centrifuged at 4°C in a SW50.1 rotor (Beckman Coulter, Fullerton, CA) for 12 h at 40,000 rpm (Tanese, 1997). Ten 0.5-ml fractions were collected from the top of the tube and analyzed by immunoblotting using rabbit anti–ART-27 or anti-TBP mouse monoclonal antibody.

RESULTS

Cloning and Characterization of ART-27

To identify proteins that interact with the AR N terminus, we elected to screen for interacting proteins using a yeast two-hybrid system. However, the AR N terminus shows strong transcriptional activity in yeast when fused to the LexA DNA-binding domain, making it unsuitable as a bait in a conventional yeast two-hybrid screen. Therefore, we modified the two-hybrid system to allow for the selection of proteins that interact with transcriptional activators. With this approach, the AR N-terminal bait is created by linking amino acids 18–500 to a heterologous activation domain. The prey is created by fusing a cDNA library to a DNA-binding domain rather than an activation domain as done in a conventional yeast two-hybrid system. An androgen-stimulated LNCaP prostate cancer cell cDNA library fused to the LexA DNA-binding domain was screened for proteins that interact with the AR N terminus, expressed as a galactose-inducible fusion protein linked to the B42 activation domain. Library proteins that interact with the AR N terminus will serve to reconstitute transcriptional activity, stimulating expression of Lex-responsive *Leu2* and β-galactosidase reporter genes. However, because some library plasmids may posses intrinsic activation potential, rendering them transcriptionally active when bound to DNA, a second screen was performed to eliminate these self-activating false positives by replica plating the $Leu2^+/LacZ^+$ colonies onto glu-

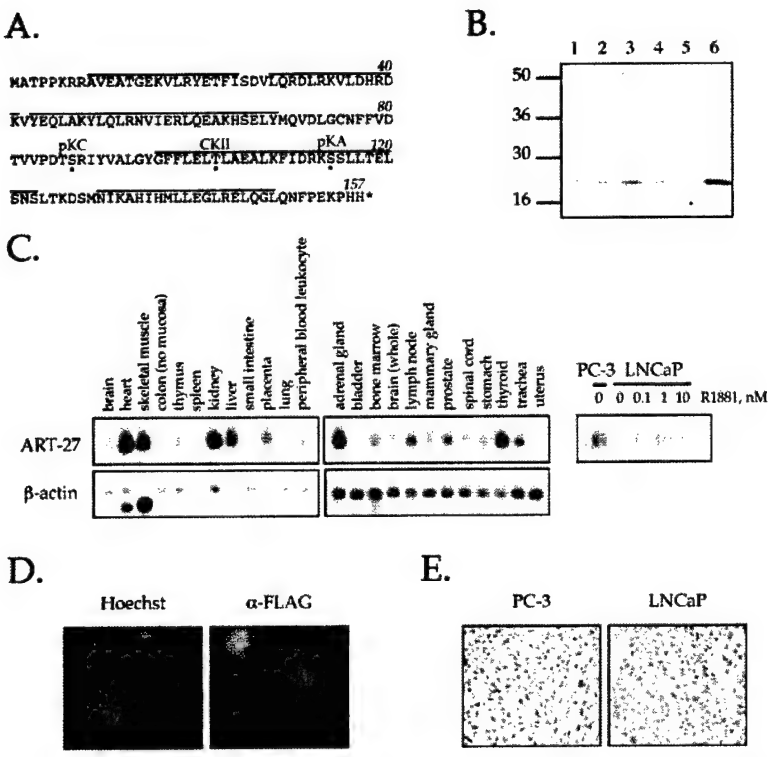

Figure 1. Cloning and characterization of ART-27. (A) Amino acid sequence of ART-27. The amino acid sequence of ART-27 is shown with an asterisk, representing the stop codon. Potential phosphorylation sites for protein kinase C (pKC), casein kinase II (CKII), and protein kinase A (pKA) are marked by a dot below the target residue. Lines above the sequence represent predicted α-helical regions as determined by Chou-Fasman and Garnier-Osguthorpe-Robson algorithms. (B) Expression of ART-27 protein. Equal amounts (50 μg) of nuclear extracts prepared from HeLa cells (lane 1), treated with TPA for 2 h (lane 2), serum-starved and stimulated with serum for 2 h (lane 3), PC-3 cells (lane 4), and whole cell extracts from COS1 cells transfected with pcDNA3 (lane 5) or pcDNA3:ART-27 (lanes 6) were analyzed by immunoblotting with affinity-purified anti–ART-27 antibody. No ART-27 immunoreactivity is observed with preimmune serum (Markus, Taneja, Logan, Li, Ha, Hittelman, Rogatsky, and Garabedian, unpublished results). (C) ART-27 mRNA expression in human tissues and prostate cancer cell lines. A human multiple tissue Northern blot (CLONTECH: human 12-lane MTN Blot I and II) containing 2 μg of poly(A+) mRNA from the indicated tissues was hybridized with ^{32}P-labeled probes corresponding to ART-27 (top) and β-actin (bottom). Total RNA was extracted from PC-3 and LNCaP cells cultured in the absence or presence of the indicated doses of androgen (R1881) for 72 h. Equal amounts of RNA were separated on denaturing formaldehyde-agarose gels (see MATERIALS AND METHODS), transferred to a Duralon nylon membrane, and hybridized to ^{32}P-labeled cDNA probes corresponding to ART-27 (right). Equal loading for each lane was determined by ethidium bromide staining of the 28S rRNA (Markus, Taneja, Logan, Li, Ha, Hittelman, Rogatsky, and Garabedian, unpublished results). (D) ART-27 subcellular localization. HeLa cells were transfected with an FLAG–ART-27 expression construct, fixed, permeabilized, and incubated with an anti-FLAG primary antibody and a corresponding fluorescein-conjugated secondary antibody, and the DNA in nucleus was stained with Hoechst dye H334211. Cells were visualized using a Zeiss Axioplan 2 fluorescence microscope. No signal above background was observed when the primary antibody was omitted (Markus, Taneja, Logan, Li, Ha, Hittelman, Rogatsky, and Garabedian, unpublished results) or in nontransfected cells. Note that the FLAG–ART-27 fluorescence is localized predominantly to the nucleus. (E) Expression pattern of endogenous ART-27 in human PC-3 and LNCaP prostate cancer cell xenografts. Immunohistochemical analysis of paraffin-embedded PC-3 (left) and LNCaP (right) human prostate cancer cell xenografts hybridized with affinity-purified ART-27 antibody is shown (200×). Immunoreactivity, seen as brown staining within the nuclei of these cells, is blocked by coincubation of the antibody with the ART-27 peptide immunogen (Markus, Taneja, Logan, Li, Ha, Hittelman, Rogatsky, and Garabedian, unpublished results).

cose plates, conditions in which the AR protein is not expressed. Clones that activated the β-galactosidase reporter gene on both glucose and galactose plates were discarded as false positives. Colonies that activated β-galactosidase expression on galactose plates, when the AR N terminus is expressed, but not glucose plates, were deemed true interactors and were further analyzed.

Several positive clones were identified, one of which was termed ART-27 (androgen receptor trapped clone 27). The clone from the yeast two-hybrid assay corresponded to the full-length cDNA and contained an insert of ~900 bp, in which the largest open reading frame (ORF) encoded a protein of 157 amino acids with an estimated size of 18 kDa. A BLAST search of the GenBank database revealed that ART-27 is located on the X chromosome (Xp11.23–11.22) and is identical to a recently identified ORF of unknown function, termed ubiquitously expressed transcript (UXT; accession no. AF092737), which is prevalent in tumor tissues (Schroer *et al.*, 1999). The ART-27 protein contains potential phosphorylation sites for protein kinase A, protein kinase C and casein kinase II but no other obvious motifs as determined by PROSITE (Figure 1B; Hofmann *et al.*, 1999). Secondary structure prediction algorithms suggest that ART-27 is composed of multiple, successive α helices (Chou and Fasman, 1978; Garnier *et al.*, 1978). ART-27 also appears to be conserved throughout evolution, with model organisms *Mus musculus* (accession no. AF092738), *Arabidopsis thaliana* (accession no. AC006535), *Drosophila melanogaster* (accession no. AE003412), and *Caenorhabditis elegans* (accession no. U40934), displaying 79, 55, 49, and 26% identity, respectively, with human ART-27. Interestingly, ART-27 showed no significant homology to *Saccharomyces cerevisiae* proteins, suggesting that ART-27 first arose in metazoans, as did nuclear receptors (Amero *et al.*, 1992; Owen and Zelent, 2000).

A polyclonal antibody was made against the ART-27 C terminus. Immunoblot analysis of HeLa and PC-3 cell nuclear extracts show that the antibody recognizes a single endogenous protein with an estimated molecular mass of ~18 kDa that migrates at the same apparent molecular weight as the cloned ART-27 expressed in COS-1 cells (Figure 1B, compare lanes 1, 4, and 6), confirming that the ORF

predicted from sequence analysis was indeed ART-27. No difference in ART-27 expression was observed between untreated and TPA-treated HeLa nuclear extracts (Figure 1B, compare lanes 1 and 2), suggesting that activation of protein kinase C does not affect ART-27 expression or electrophoretic mobility indicative of phosphorylation. However, a slight increase in ART-27 expression was observed in serum-stimulated HeLa nuclear extracts as compared with untreated control cells (Figure 1B, compare lanes 1 and 3), suggesting that ART-27 expression may be regulated by extracellular signals or through the induction of cellular proliferation.

Northern hybridization analysis was carried out to determine the expression pattern of ART-27 mRNA in human tissues. A single transcript of ~0.9 kb is present at variable levels in the human tissues examined, with the highest levels in the heart, skeletal muscle, kidney, liver, adrenal gland, lymph node, prostate, and thyroid and the lowest levels in bladder and uterus. We also performed Northern blot analysis on mRNA isolated from androgen-independent (PC-3) and androgen-dependent (LNCaP) prostate cancer cells, either untreated or treated for 72 h with the synthetic androgen R1881 at the indicated concentrations (Figure 1C). ART-27 steady-state mRNA expression is slightly higher in PC-3 relative to LNCaP cells and is weakly induced in LNCaP cells in response to androgen.

To investigate the subcellular localization of ART-27, we performed indirect immunofluorescence on HeLa cells transfected with FLAG-tagged ART-27. FLAG–ART-27 localized predominantly to the nucleus, although some diffuse staining was apparent in the cytoplasm of cells expressing high levels of the protein (Figure 1D). Immunohistochemical staining with affinity-purified ART-27 antibody performed on sections of human PC-3 and LNCaP cell xenograft tumors demonstrated strong nuclear staining for endogenous ART-27 (Figure 1E); staining was blocked by coincubation of the antibody with the ART-27 peptide immunogen (Markus, Taneja, Logan, Li, Ha, Hittelman, Rogatsky, and Garabedian, unpublished results), demonstrating the specificity of the antibody-antigen interaction. Interestingly, ART-27 appears to be expressed in a subset of cells from the xenografts. Although we did not investigate the reason for this heterogeneous staining of ART-27, it may reflect cell cycle regulation or other cellular parameters. Regardless, such predominant nuclear distribution of ART-27 is consistent with its role as a putative transcriptional regulatory protein.

ART-27 Interacts with AR In Vitro and in Cell Extracts

To confirm the results of the two-hybrid screen by an independent biochemical method, we examined whether AR and ART-27 proteins bind tightly enough to each other to be coimmunoprecipitated and whether the interaction was affected by androgen treatment. The ability of ART-27 and AR to interact was first tested in vitro. Full-length AR and HA–ART-27 were expressed in a coupled transcription/translation system and immunoprecipitated with an antibody against the HA epitope on ART-27. In vitro translated AR was precipitated with the HA antibody in the presence, but not in the absence, of HA–ART-27 in a hormone-independent manner (Figure 2A). We next tested the ability of ART-27 to interact with endogenous AR in LNCaP cells that

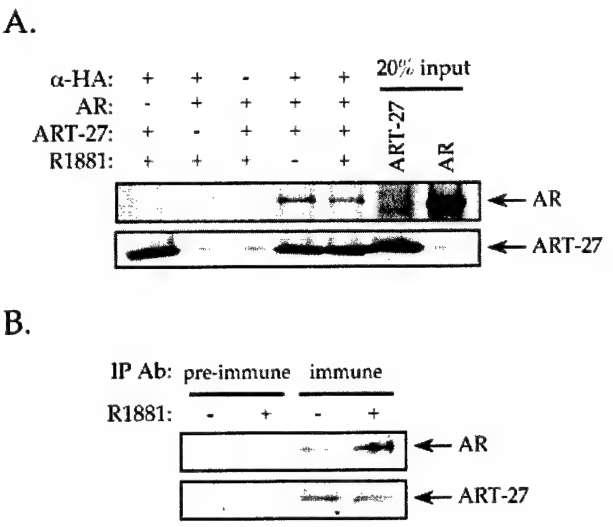

Figure 2. ART-27 interacts with AR in vitro and in cell extracts. (A) Full-length human AR and HA–ART-27 were synthesized in vitro using a coupled transcription/translation system in the presence of [^{35}S]methionine and in the absence or presence of 100 nM R1881, as indicated, and incubated with an antibody against HA. Bound proteins were collected on Protein A Sepharose beads, washed, eluted, resolved by SDS-PAGE, and visualized by autoradiography. Twenty percent of the input AR and ART-27 translation reaction are shown. (B) ART-27 interacts with endogenous AR in LNCaP cell extracts. Nuclear extracts from untreated and 100 nM R1881-treated LNCaP-Tet-on-ART-27-FLAG cells were prepared as described in MATERIALS AND METHODS and incubated with an antibody against either preimmune or ART-27 immune sera. Immune complexes were collected on protein A Sepharose beads, washed, eluted, resolved by SDS-PAGE, and transferred to a membrane. The filter was probed with antibody against AR and ART-27.

express ART-27 in an inducible manner. Nuclear extracts from untreated or R1881-treated LNCaP cells were subjected to immunoprecipitation with anti–ART-27 antibody and analyzed by immunoblotting with antibodies to AR and ART-27. AR was coimmunoprecipitated by the ART-27 antibody but not by preimmune serum in both the absence and presence of hormone, with slightly more AR found in association with ART-27 upon hormone treatment (Figure 2B). As expected, ART-27 was also immunoprecipitated by the anti–ART-27 antibody in either the presence or absence of hormone (Figure 2B). These results demonstrate the presence of an AR–ART-27 complex in LNCaP cells and substantiate the interaction observed in the yeast two-hybrid system.

ART-27 Interaction Specificity

To analyze the specificity of AR:ART-27 interaction, we examined the capacity of ART-27 to associate with a panel of transcriptional regulatory proteins in the yeast two-hybrid system, including the AR C-terminal ligand-binding domain (LBD), the AF-1 region of the GR, ERα, the steroid receptor coactivator-1 (SRC-1), the TBP-associated factor 130 ($TAF_{II}130$), the Sp1 (A and B domains), the cyclic AMP response element-binding protein (CREB), and VP16. As shown in Figure 3, ART-27 interacts with the AR N terminus

Regulatory Factors	ART-27 interaction strength
AR N-T	10,000
AR LBD	1
GR AF-1	14
GR 30IIB	8
GR LBD	1
ER α	19
TAF130	3,374
SRC-1	1
CREB-N	1
Sp1A	19
Sp1B	264
VP16	1

Figure 3. Specificity of AR:ART-27 interactions. Interaction of ART-27 with the AR N terminus (N-T; rat AR_{18-500}), AR LBD (rat $AR_{579-901}$), and other transcriptional regulatory factors was analyzed using the modified yeast two-hybrid assay. Regulatory factors examined include the GR AF-1 (rat $GR_{107-237}$), GR 30IIB (rat $GR_{107-237}$ E219K, F220L, W234R), SRC-1 ($SRC\text{-}1_{374-800}$), GR LBD (rat $GR_{525-795}$), $TAF_{II}130$ ($TAF_{II}130_{270-700}$), Sp1 A ($Sp1_{83-262}$), Sp1 B ($Sp1_{263-524}$), CREB-N ($CREB_{3-296}$), ERα($ER\alpha_{1-595}$), and VP16. The strength of interaction was determined by a quantitative liquid β-galactosidase assay after a 24-h incubation in galactose medium at 30°C and normalized to protein expression using the common HA epitope resident on each protein. The background from the vector-only control was subtracted from each sample. The interaction with ERα was observed in the presence, but not the absence (Markus, Taneja, Logan, Li, Ha, Hittelman, Rogatsky, and Garabedian, unpublished results), of 100 nM 17β-estradiol. No interaction was observed with AR LBD and the GR LBD in either the presence or absence of 100 nM R1881 and 10 μM deoxycorticosterone, the preferred GR ligand in yeast (Garabedian and Yamamoto, 1992).

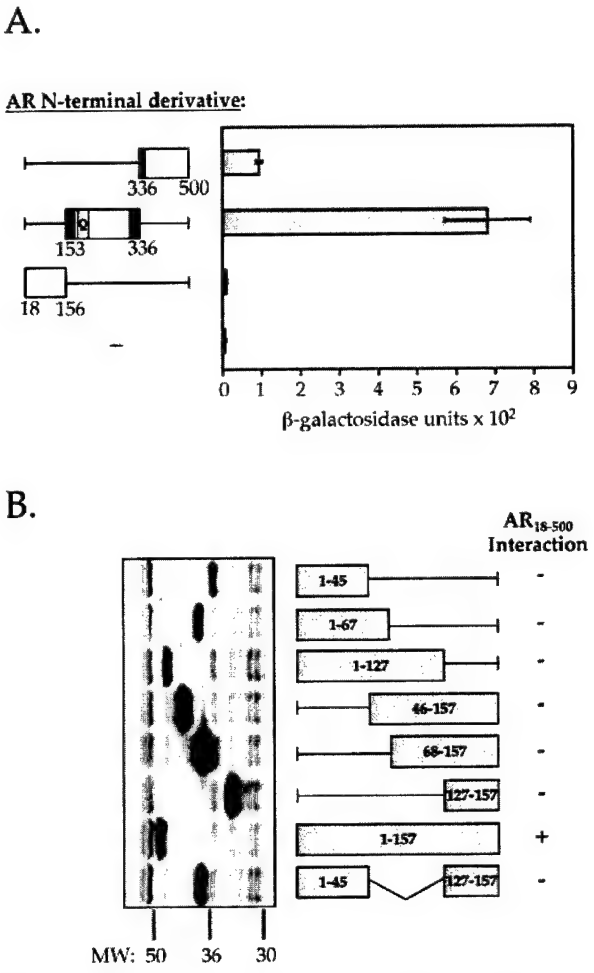

Figure 4. Domains of AR and ART-27 mediating interaction. (A) Quantitative analysis of ART-27 interaction with AR N-terminal derivatives. The relative affinity of ART-27 for the AR N-terminal subdomains 336–500, 153–336, and 18–156 was assessed using the yeast two-hybrid assay. The dark gray boxes show the location of the AR N-terminal activation functions AF-1a and AF-1b and the light gray box denotes the glutamine (Q) repeat region. Data represent the means of triplicate data points normalized to cell number. (B) Only full-length ART-27 interacts with the AR N terminus. ART-27 deletion derivatives fused to LexA were tested for their ability to interact with the AR N terminus (AR_{18-500}) using a qualitative β-galactosidase plate assay after a 24-h incubation on galactose X-gal plates at 30°C. +, strong interactions (blue colonies); −, no interactions above background vector-only control (white colony). Left, an immunoblot of the ART-27 derivatives expressed in yeast and probed with an antibody against the LexA moiety common to all ART-27 truncations.

and weakly with the GR N terminus, as well as with ERα, Sp1, and $TAF_{II}130$ (rank order of interaction: AR N $terminus_{18-500}$ > $TAF_{II}130$ > Sp1B > ERα = Sp1A > GR N terminus) but not with SRC-1, CREB, VP16, or the AR or GR LBD in either the absence or presence of hormone. Proteins that did not interact with ART-27 have been shown to interact with other factors, suggesting that the lack of interaction with ART-27 is genuine. For example, SRC-1 has been shown to interact with ERα, whereas the AR and GR LBDs associate with GRIP-1, and CREB interacts with $TAF_{II}130$ in the yeast two-hybrid system (Shibata *et al.*, 1997; Saluja *et al.*, 1998; Hong *et al.*, 1999). Thus, our results indicate that ART-27 interacts not only with the AR N terminus but also with at least two other SRs and with certain other transcriptional regulators, including $TAF_{II}130$.

ART-27 Interacts with $AR_{153-336}$

To characterize the interaction of AR with ART-27 in more detail, various AR N-terminal deletion constructs were produced and their ability to interact with ART-27 was tested in the yeast two-hybrid assay. As shown in Figure 4A, ART-27 interacts with two different regions of AR: the central region of the AR N terminus ($AR_{153-336}$), encompassing all of AF-1a (residues 154–167), and a small part of AF-1b (residues 295–459), and it interacts strongly with ART-27; a weak interaction between ART-27 and the $AR_{336-500}$ subdomain was also observed, whereas no interaction was detected between ART-27 and AR_{18-156}. Immunoblot analysis of the AR_{18-156}, $AR_{153-336}$, and $AR_{336-500}$ derivatives indicates that

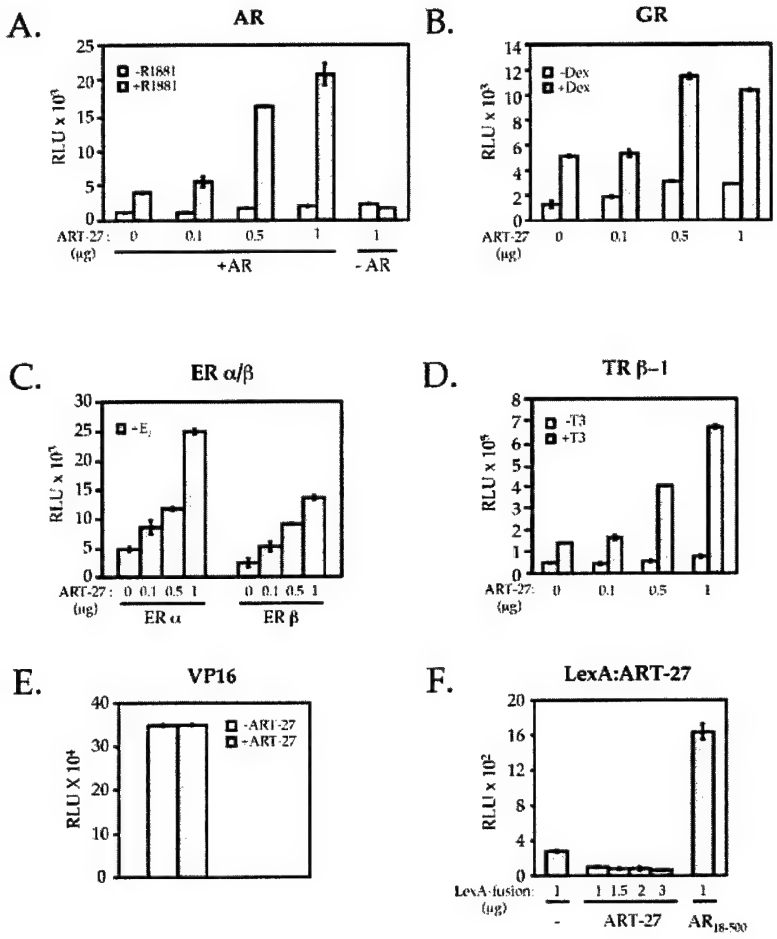

Figure 5. ART-27 enhances steroid and thyroid receptor-dependent transcriptional activation. HeLa cells (1.2×10^5 cells/35-mm dish) were transiently transfected using Lipofectamine with paired receptor (0.2 μg/dish) and reporter constructs (0.1 μg/dish) for hAR + MMTV-Luc (A), GR + MMTV-Luc (B), ERα and ERβ + XETL (C), and human TRβ-1 + pGL3-DR4 (D) along with the indicated amount of ART-27 or empty expression vector to equalize the total amount of DNA per dish and 0.05 μg of pCMV-LacZ per dish as an internal control for transfection efficiency. Cells were treated with 100 nM R1881 (A), 100 nM dexamethasone (Dex; B), 10 nM 17β-estradiol (E2; C), 100 nM triac (T3, D), or the ethanol vehicle (white bars) for 12 h, and receptor transcriptional activation was assayed as described in MATERIALS AND METHODS, normalized to β-galactosidase activity, and expressed as relative luminescence units (RLU). The average of three independent experiments and SE is shown. (E) ART-27 does not affect transactivation by VP16. HeLa cells were transfected as above with an expression construct for GAL4-VP16 and a reporter plasmid containing five Gal4-binding sites upstream of the E1b promoter, in the absence (white bars) or presence (gray bars) of 1 μg of ART-27, and assayed for luciferase activity. (F) ART-27 failed to activate transcription when tethered to DNA. HeLa cells were transfected as above with expression constructs for LexA, LexA–ART-27, or LexA-AR_{18-500} and the pΔ4X-LALO reporter plasmid and assayed for luciferase activity.

they are expressed at similar levels (Markus, Taneja, Logan, Li, Ha, Hittelman, Rogatsky, and Garabedian, unpublished results). These findings suggest that the $AR_{153-336}$ region is the primary interaction site for ART-27.

In an attempt to localize the region of ART-27 that interacts with the AR N terminus, we created a series of ART-27 deletion derivatives. ART-27 derivatives containing amino acids 1–45, 1–67, 1–127, 46–157, 68–157, 127–157, 1–157, and 1–45/127–157 were expressed as fusion proteins with LexA and analyzed for their ability to interact with AR_{18-500} in the modified yeast two-hybrid assay. Surprisingly, none of the deletion derivatives interacted with AR_{18-500} (Figure 4B), even though all of them were expressed (Figure 4B, left). This result suggests that either ART-27 requires multiple contacts for interaction with the AR N terminus or that the entire protein is involved in configuring a functional AR-interacting surface.

ART-27 Is Involved in Steroid and Thyroid Hormone Receptor Transcriptional Activation

A transient transfection assay was used to examine the role of ART-27 in the regulation of AR transcription activation. AR-deficient HeLa cells were transfected with a constant amount of full-length AR and increasing concentrations of an expression vector encoding an HA–ART-27 along with an AR-responsive luciferase reporter gene. As shown in Figure 5A, the hormone-induced AR transcriptional activation was increased in a dose-dependent manner by overexpressed ART-27. This effect was AR-dependent, because in the absence of AR, ART-27 did not influence reporter gene activity (Figure 5A). This enhanced transcriptional activity did not result from increased AR protein production, because AR levels were not affected by ART-27 coexpression (Markus, Taneja, Logan, Li, Ha, Hittelman, Rogatsky, and Garabedian, unpublished results). The effect of ART-27 on AR was not restricted to a single cell type, because overexpression of ART-27 in PC-3 and COS-1 cells also increased AR transcriptional activity in a dose-dependent manner (Markus, Taneja, Logan, Li, Ha, Hittelman, Rogatsky, and Garabedian, unpublished results). Thus, ART-27 can act as a positive regulator of AR transcriptional activity in mammalian cells.

The ability of ART-27 to affect transactivation by other members of the SR family, i.e., GR, ERα, ERβ, and the TRβ-1, was tested using transient transfection assays. Our results indicate that ART-27 increased the transcriptional activity of all four receptors in a dose-dependent manner (Figure 5,

B–D). We next tested the effect of ART-27 on VP16-dependent transactivation. Recall that VP16 did not interact with ART-27 in the yeast two-hybrid assay. Consistent with this lack of interaction, ART-27 expression had no effect on GAL4-VP-16 activity from a reporter plasmid containing five Gal4-binding sites upstream of the E1b promoter (Figure 5E). Together, these results suggest that ART-27 increases transactivation by steroid and thyroid hormone receptors.

We also examined the ability of ART-27 to activate transcription when artificially recruited to promoters in mammalian cells. Our results indicate that recruitment of ART-27 to a promoter by fusing it to either the Gal4 (Markus, Taneja, Logan, Li, Ha, Hittelman, Rogatsky, and Garabedian, unpublished results) or LexA (Figure 5F) DNA-binding domain fails to activate transcription, even though the proteins were expressed (Markus, Taneja, Logan, Li, Ha, Hittelman, Rogatsky, and Garabedian, unpublished results). In fact, transcriptional activation of the LexA–ART-27 derivative was slightly reduced relative to LexA, suggesting that ART-27 may interact with and sequester factor(s) responsible for "basal" promoter activity. Thus, ART-27 lacks an intrinsic transactivation function. This may suggest that ART-27 is unable to overcome a rate-limiting step in transcription when artificially recruited to a promoter or, alternatively, that ART-27 is only one target of the AR and that the receptor requires multiple targets to recruit mammalian Pol II and initiate transcription.

Enhanced AR-dependent Transcriptional Activation by ART-27 Is Mediated Through a Distinct Receptor N-Terminal Domain

Because ART-27 interacts strongest with the AR subdomain spanning amino acids 153–336 (Figure 4A), we expect that it would affect the transcriptional activation potential of this AR subdomain. To determine whether ART-27 could affect the function of the different AR subdomains, AR N-terminal derivatives containing amino acids 18–156, 153–336, 336–500, and 18–500 were expressed as fusion proteins with the LexA DNA-binding domain. HeLa cells were transfected with the LexA-AR N-terminal derivatives, along with a LexA-responsive luciferase reporter gene in the presence and absence of ART-27. In the absence of ART-27 coexpression, all four subdomains of the AR N terminus are capable of activating transcription of the LexA-luciferase reporter gene to varying degrees (Figure 6A). Importantly, overexpression of ART-27 enhances the transcriptional activity of two AR derivatives containing the ART-27 interaction region, LexA-$AR_{153-336}$ and Lex-AR_{18-500}, but not that of the derivatives lacking this region, LexA-AR_{18-156} and LexA-$AR_{336-500}$. In fact, transcriptional activation of the LexA-$AR_{336-500}$ derivative was slightly reduced by ART-27 overexpression, suggesting that ART-27 may interact with and sequester a factor responsible for AR transactivation via the 336–500 subdomain. Immunoblotting with an antibody against the LexA moiety common to all derivatives indicates that expression of these chimeras is unaffected by coexpression of ART-27 in HeLa cells (Figure 6B). These results suggest that the enhancement of AR transcriptional activation by ART-27 is mediated via the ART-27–interacting region.

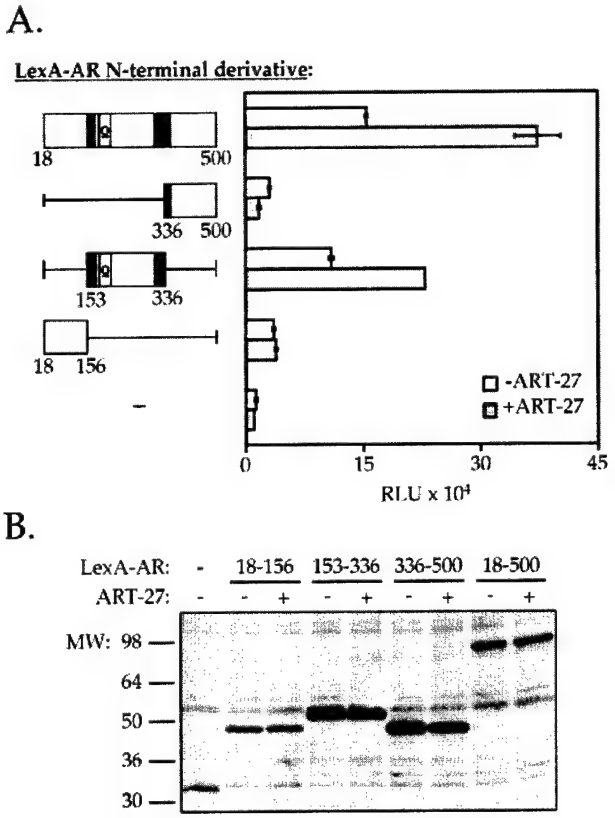

Figure 6. The effect of ART-27 on AR transcriptional activation is dependent on the AR-interacting region. (A) HeLa cells were transfected with 0.5 μg of the pcDNA3LexA:AR N-terminal derivatives indicated and either 1.0 μg of the empty expression vector (white bars) or 1.0 μg pcDNA3-HA–ART-27 (gray bars) along with 1.0 μg of the LexA responsive-luciferase reporter (pΔ4X-LALO) and 0.25 μg of pCMV-LacZ, and AR activity was determined as described in Figure 5. Data represent the means of duplicate data points normalized to β-galactosidase units. Error bars represent the range of the duplicate data points. (B) The expression of the LexA:AR derivatives was analyzed by immunoblotting from a parallel set of transfections using a polyclonal antibody to LexA.

ART-27 Is Part of a High Molecular Weight Complex

Several transcriptional regulatory cofactors have been identified as components of multiprotein complexes. We therefore examined whether ART-27 is part of a higher-order species using velocity gradient sedimentation. HeLa cell nuclear extracts were applied to 15–35% glycerol gradients, either containing or lacking 2.4 M urea. After centrifugation, fractions were collected and analyzed by immunoblotting with antibodies specific for ART-27 or TBP. Previous work has shown that TAFs and TBP exist in a complex, which can be dissociated with urea (Tanese *et al.*, 1991). It has been shown that the larger, faster sedimenting species corresponds to TBP and TAFs binding together as TFIID, whereas the smaller, slower migrating peak represents additional non-TFIID TBP complexes (Tanese *et al.*, 1991). As expected, under native conditions, TBP sedimented at the bottom, in

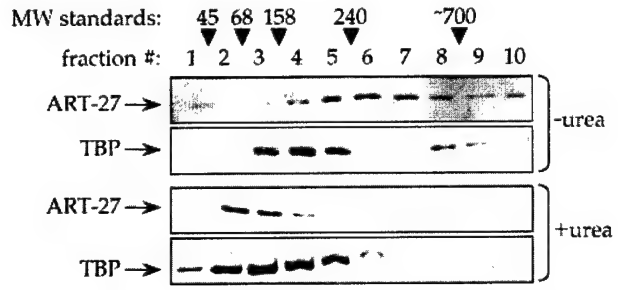

Figure 7. Native ART-27 is part of a high molecular weight complex. Nuclear extracts were prepared from HeLa cells and loaded onto linear 15–35%glycerol gradients with or without 2.4 M urea. Fractions were collected and analyzed by Western blot using affinity-purified ART-27 polyclonal or TBP monoclonal antibodies. Migration of molecular mass markers is indicated: 45- and 68-kDa albumin, 158-kDa aldolase, 240-kDa catalase. The molecular mass estimate of TFIID is 700 kDa (Tanese *et al.*, 1991).

the high-density region of the gradient in two discrete peaks. Under native conditions, a majority of ART-27 also migrated within the high-density region of the gradient (estimated range of molecular mass between 240 and 700 kDa; Figure 7). In the presence of 2.4 M urea, the sedimentation patterns of TBP and ART-27 were shifted to the top of the gradient (Figure 7). The change in mobility of TBP is in agreement with previous results demonstrating the sedimentation profile of dissociated TFIID (Tanese *et al.*, 1991). These results are consistent with the idea that ART-27 is part of a high molecular weight complex, the components of which have yet to be identified.

DISCUSSION

We have identified ART-27 as a protein that interacts with the AR N-terminal subdomain spanning amino acids 153–336, including AF-1a (154–167) and a part of AF-1b (295–459), and enhances AR transcriptional activation when overexpressed in mammalian cells. The ability of ART-27 to affect AR transcriptional activation is dependent on the ART-27:AR–interacting region, because only the AR N-terminal derivatives containing the interaction domain are enhanced by ART-27 coexpression. Thus, ART-27 represents a novel AR N terminus-associated coactivator.

ART-27 was originally identified as a novel transcript in a screen for genes that map to the human Xp11 locus, a region previously shown to contain an abundance of disease loci (Schroer *et al.*, 1999). However, the designation of ART-27 as a ubiquitously expressed transcript is misleading because the level of ART-27 mRNA varies widely among tissues examined (Figure 2C). Interestingly, ART-27 and AR reside in an amplicon found in a subset of hormone-refractory prostate cancers, suggesting that ART-27 may play a role in AR-dependent prostate tumorigenesis (Visakorpi *et al.*, 1995a,b).

Although we have localized the region of the AR N terminus (residues 153–336) necessary for interaction with ART-27, homology searches among the other receptors affected by ART-27 have not revealed a common ART-27 interaction motif. We are currently testing whether single amino acid substitutions in the AR N terminus that have been implicated in androgen insensitivity syndrome affect interaction with ART-27. The amino acid alternations in the AR N terminus identified in androgen insensitivity syndrome patients likely represent AR loss-of-function mutations, which have reduced AR activity possibly because of the loss of receptor-cofactor interactions. This approach might help pinpoint the residues of AR that interact with ART-27, which in turn will help define the corresponding sites in the other receptors.

Deletion analysis of ART-27 failed to define a distinct interaction surface within ART-27 that mediates association with the receptor. One plausible explanation is that ART-27 may associate with the AR N terminus through multiple low-affinity interactions, and removal of any one of these contacts renders ART-27 incapable of binding. Alternatively, the entire ART-27 may be involved in configuring a functional protein and its integrity may be compromised upon deletion of any region. Whether ART-27 contains independent interaction surfaces for AR or consists of multiple regions that function together to coordinate the tertiary structure of the protein will require a detailed structure-function analysis, which is currently underway.

The ART-27 primary amino acid sequence as well as the predicted secondary structure composed of tandem α-helices appears conserved from worms to humans. Although the function of the ART-27 orthologs has yet to be determined, our findings suggest a link to nuclear receptor function as a potential coactivator protein. Recently, the *taiman* gene of *Drosophila*, a protein involved in cell migration, was found to be homologous to the mammalian p160 coactivator AIB1 and to function as a coactivator for the ecdysone receptor (Bai *et al.*, 2000). Thus, like *taiman*, ART-27 may represent an evolutionarily conserved transcriptional cofactor that regulates a wide array of responses controlled by nuclear receptors.

The mechanism by which ART-27 affects AR-mediated transcriptional activation remains to be defined. ART-27, a comparatively small protein with a predicted molecular mass of ~18 kDa, has little transcriptional activation ability when tethered to DNA, suggesting that it does not harbor an intrinsic transactivation function. This may seem unusual for a transcriptional cofactor; however, recent studies by Dorris and Struhl (2000) demonstrate that several components of the RNA Pol II holoenzyme are not transcriptionally active when tethered to DNA. Because ART-27 migrates in velocity gradient sedimentation analysis as a large molecular weight species (Figure 7), we speculate that ART-27 may represent a subunit of a previously characterized (such as DRIP/TRAP/ARC; Fondell *et al.*, 1996; Rachez *et al.*, 1998, 1999; Naar *et al.*, 1999) or novel multiprotein coactivator complex. Our preliminary findings suggest that the purified DRIP complex does not contain an ART-27 immunoreactive species by Western blot, suggesting that ART-27 is not part of the DRIP complex (S. M. Markus, L.P. Freedman, and M.J. Garabedian, unpublished observation). ART-27 also interacts with $TAF_{II}130$ in the yeast two-hybrid assay, suggesting that ART-27 may communicate with at least one other transcriptional regulatory cofactor. Interestingly, $TAF_{II}130$ itself appears to interact with and increase transcriptional activation of AR via a distinct N-terminal subreigon (Taneja and Garabedian, unpublished observation). Additional studies

are needed to determine the components of this putative ART-27–containing complex as well as the mechanism of ART-27 function.

The AR N terminus appears to be a multifaceted platform capable of interacting with a variety of transcriptional regulatory proteins, including ART-27, which likely collaborate to regulate gene- and tissue-specific responses to AR. Consistent with this notion, the coactivators SRC-1, GRIP-1, and CBP have recently been shown to interact with the AR N terminus and modulate its activity (Ikonen *et al.*, 1997; Alen *et al.*, 1999; Bevan *et al.*, 1999; Ma *et al.*, 1999). Although ART-27 is expressed in a wide variety of tissues, distinct cofactor combinations in target tissues may result in cell-specific regulation of AR. Thus, based on its nuclear localization, its binding to the AR N terminus, and its ability to potentiate steroid and thyroid hormone receptor-dependent transcription in cultured cells, ART-27 represents a new class of coactivator proteins.

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Androgen receptor coactivator ART-27, in androgen-mediated cell growth, cancer and development

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Abbreviations: AF-1, activation function 1, AF-2, activation function 2, AR, androgen receptor, ART-27, Androgen Receptor Trapped clone-27, ARE, androgen response element, Dox, doxycycline, IRES, internal ribosome entry site, PB, prostatic bud, PCNA, proliferating cell nuclear antigen, PIN, prostatic intraepithelial neoplasia, TBP, TATA box binding protein

Abstract

Androgen Receptor Trapped clone-27 (ART-27) is a newly described transcriptional coactivator that binds to the N-terminus of the androgen receptor (AR). Given the vital importance of AR signaling in prostate growth and differentiation, we investigated the role of ART-27 in these processes. Immunohistochemical studies indicate that ART-27 protein is expressed in differentiated epithelial cells of adult human prostate and breast tissue. In prostate, ART-27 is abundant in AR positive prostate luminal epithelial cells, in contrast to the stroma, where cells express AR but not ART-27. The use of a rat model of androgen depletion/reconstitution indicates that ART-27 expression is associated with the elaboration of differentiated prostate epithelial cells. Interestingly, regulated expression of ART-27 in the androgen sensitive LNCaP prostate cancer cell line inhibits androgen-mediated cellular proliferation. Consistent with a growth suppressive function, we show that ART-27 expression levels are negligible in human prostate cancer. Importantly, examination of ART-27 protein expression in early fetal prostate development demonstrates that ART-27 is detected only when the developing prostate gland has proceeded from a solid mass of undifferentiated cells to a stage in which differentiated luminal epithelial cells are evident. Thus, ART-27 is the first AR cofactor shown to be subject to both cell type and developmental regulation in humans. Overall, the results suggest that decreased levels of ART-27 protein in prostate cancer tissue may occur as a result of de-differentiation, and indicate that ART-27 is likely to regulate a subset of AR responsive genes important to prostate growth suppression and differentiation.

Introduction

Androgen steroid hormones direct the genetic program dictating prostate development and maturation male development. They exert biological effects by binding to the androgen receptor (AR), a member of the steroid receptor family of transcription factors. Functional mapping of the androgen receptor shows that several regions are required for transcriptional activation (1,2). These include a carboxy-terminal domain called AF-2, as well as two regions in the N-terminus called AF-1a and AF-1b. Recent evidence suggests that the AR cell- and promoter-specific transcriptional response is generated through interactions with regulatory proteins termed coactivators and corepressors with AF-1 and AF-2 (3,4) (2,5-12).

Androgen stimulation results in cell proliferation in both the developing prostate and the malignant prostate. Current therapy for metastatic prostate cancer includes injections of luteinizing hormone-releasing hormone (LHRH) analogues to pharmacologically lower testosterone levels (androgen ablation), treatment with anti-androgens such as flutamide or bicalutamide, to block testosterone binding to the AR, or a maximal androgen blockade in which both treatments are combined. While such therapies are effective in the short term, cancers treated in this fashion will inevitably progress in an androgen-independent fashion.

An important determinant of AR transactivation function resides in the N-terminus (1,13,14). The AR N-terminal residues 142-485 have been shown to activate a minimal promoter construct in a cell-free transcription system and to selectively interact with the transcription factors TFIIF and the TATA-Binding Protein (TBP), suggesting a direct contact with the general transcription factors (15,16). A growing list of proteins has also been reported to interact with the AR N-terminus (15,17-22). The clinical importance of investigating the biological role of coactivators that bind the AR N-terminus is underscored by a recent report describing a patient diagnosed with complete androgen-insensitivity, but exhibiting no mutation in AR. The studies

suggested that a coactivator interacting with the N-terminal region of AR, apparently essential for genital development, was lacking in this patient (23).

The work described in this paper investigates the biological role of the newly described androgen receptor coactivator androgen receptor trapped clone-27 (ART-27). ART-27 binds to the AR N-terminus and facilitates receptor dependent transcriptional activation. The ART-27 clone was isolated from an androgen-stimulated LNCaP cell cDNA library in a two hybrid screen designed to identify proteins that interacted with the AR-N-terminus (24) and was previously cloned in an exon trapping experiment (25). The ART-27 gene encodes a protein of 157 amino acids with a calculated molecular weight of 18 kDa. Functional characterization of ART-27 indicates that it is a nuclear protein that interacts with N-terminal rat AR amino acids 153-336, containing AF-1a and a part of AF-1b (24). Biochemical analysis of ART-27 indicates that it interacts with AR in nuclear extracts from LNCaP cells in a ligand-independent manner. In addition, ART-27 increases the transcriptional potency of the AR when over-expressed in a variety of cultured mammalian cells. Mechanistically, ART-27 is likely to function as part of a transcription complex since analysis of ART-27 behavior in density gradient sedimentation of HeLa nuclear cell extracts indicates that it co-sediments as part of a larger complex. Although the mechanism by which ART-27 functions as a transcriptional activator is unclear, it is likely that ART-27 acts as an adapter to assemble protein complexes at androgen responsive promoters.

Steroid receptor coactivators and corepressors are likely important determinants of the response of a given hormone receptor in a given cellular context. As androgen stimulation results in differing cellular responses at various stages of prostate development and malignant transformation, we investigated the role of ART-27 in prostate epithelial cells.

Materials and Methods

Construction of plasmids- The ART-27 GST fusion construct was made by isolating full length ART-27 as a EcoR1/Xho1 fragment from pEG202 and subcloning into EcoR1/Xho1 sites in pGEX-4T-1 (Amersham Biosciences). FLAG-tagged ART-27 and HA-tagged ART-27 were made by subcloning the ART-27 EcoR1/Xho1 fragment into a pcDNA3 vector with an N-terminal hemagglutinin (HA) epitope (pcDNA3-HA) or into a pcDNA3 vector with a C-terminal FLAG epitope (pcDNA3-FLAG).

Generation of anti-ART-27 polyclonal antibodies- Generation of C-terminal anti-ART-27 antibodies has been previously described (24). C-terminal antibodies were affinity purified by isolation of the IgG fraction on a Protein A Sepharose column, followed by affinity purification using Affi-gel 15 resin (Bio-Rad Laboratories, Hercules, CA) coupled to the ART-27 peptide immunogen. Antibody was eluted with 100mM glycine pH 2.7, and immediately neutralized. Antibodies against the ART-27-GST fusion protein were made by immunizing rabbits with the purified protein (Covance Research Products, Denver, PA).

Immunohistochemistry- All human samples were used with approval of the New York University School of Medicine Institutional Review Board. Tissue utilized was obtained from paraffin-embedded archived radical prostatectomy or prostate needle biopsy samples. Immunohistochemistry was preformed using either affinity purified C-terminal ART-27 antibody or AR antibody (Santa Cruz AR polyclonal N-20). Tissue sections were dewaxed in xylene, rehydrated and washed in Tris-buffered saline (TBS), pH7.4 (20mM Tris pH 7.5, 150mM

NaCl). For antigen retrieval, paraffin sections were heated in a microwave oven (900 W) in Target Retrival Solution (Dako), followed by treatment with 3% H_2O_2 and blocking with 20% normal goat serum. Sections were then incubated with affinity purified ART-27 (1:100) or AR antibody (1:200) followed by incubation with a biotinylated rabbit secondary antibody. Prostate specimens from 9 human fetuses 9.5-24 weeks of gestational age were obtained following surgical abortion performed for reasons unrelated to this investigation. Approval for the collection of specimens was obtained by the New York University School of Medicine Institutional Review Board. Informed consent was obtained for all specimens. Age of specimens were estimated by heel-toe length and based on the last menstrual period. Prostates were formalin-fixed and oriented in paraffin blocks.

Rat model of androgen depletion/reconstitution- Adult Sprague-Dawley rats (228-250 grams) were obtained from Taconic (Germantown, NY). Two control rats were sacrificed on day 0 and ventral prostates harvested. The remaining rats were anesthetized and castrated on day 0. Following this, two rat ventral prostates were harvested at 96 hours. In addition, at the 96 hour post-castration time point, the remaining rats were implanted with 25mg, 21-day release testosterone pellets (Innovative Research of America, Sarasota, FL). Two ventral prostates were then harvested at 24 and 48 hours following testosterone supplementation. The weights of the prostates indicated that tissue volume substantially diminished upon androgen depletion.

Generation of tet-ART-27 cells- Tetracycline inducible cell lines expressing a FLAG epitope tagged ART-27 gene were made in LNCaP cells. LNCaP cells were first transfected with the pTet-On vector using DOTAP (Roche, Summerville, NJ) and resistant colonies were selected in 500 μg/ml Geneticin (Invitrogen). Clones were transferred to 24 well dishes coated with fibronectin (10 μg/ml; Invitrogen), expanded and screened for Tet-dependent activation by measuring pRevTRE-luciferase reporter gene activity. A LNCaP clone displaying tight Tet-dependent activation was transfected with pRevTRE:ART-27 (C-FLAG) prior to selection of resistant colonies in 150μg/ml hygromycin B. Approximately 70 clones were expanded and grown in the presence and absence of doxycycline. Cell lysates were made and subjected to Western blot analysis to determine which cell lines showed doxycycline regulated ART-27 FLAG expression. Anti-FLAG antibody (Sigma) was used for Western blot analysis.

Thymidine Incorporation- LNCaP cell clones were plated in 24 well plates in RPMI 1640 + 10% FBS. The following day (T=0), cells were washed with PBS and media changed to RPMI 1640 (without phenol red) + 10% charcoal stripped FBS (cFBS) +/- 1μg/ml doxycycline and +/- R1881. Media was changed every 24 hours during the duration of the experiment. H^3-thymidine (2μCi) was added to fresh media and incubated with cells for 1 hour at 37° C. Cells were then washed, methanol fixed, and solublized prior to scintillation counting.

Results

ART-27 is expressed in epithelial cells of human prostate and breast tissues

To evaluate the biological role of ART-27, polyclonal antibodies were developed so that the cell and tissue specific expression pattern of ART-27 could be determined *in vivo*. Characterization of anti-ART-27 polyclonal antibodies is shown in Figure 1. Cells were transfected with tagged ART-27 and lysates were either immunoprecipitated with antibody

against ART-27 or the tag or used directly for Western blot analysis. Figure 1A (top panel) shows that a polyclonal antibody made against the C-terminal peptide of ART-27 recognizes over-expressed FLAG-tagged ART-27 (lanes 1 and 5), over-expressed HA-tagged ART-27 (lanes 2 and 6) as well as endogenous ART-27 protein (lanes 1, 2, 3, 4 and 7). A second polyclonal antibody made against full length ART-27-glutathione S-transferase (gst) protein similarly recognizes these proteins (Figure 1A bottom panel) and confirms the identity of ART-27 protein. To verify detection of endogenous ART-27 protein, an untagged variant of ART-27 was expressed and compared to expression of endogenous ART-27 (Figure 1B). The results establish that endogenous ART-27 is the same size as the untagged ART-27 and that the antibody is capable of specifically recognizing over-expressed and endogenous ART-27.

To determine the cell type specificity of ART-27 expression compared with AR expression, ART-27 and AR antibodies were separately incubated with freshly cut paraffin sections of human prostate and breast tissue. Consecutive sections from each tissue were analyzed. Both ART-27 and AR are predominantly expressed in nuclei of differentiated epithelial cells of human prostate tissue. AR is almost exclusively nuclear, while ART-27 immunoreactivity appears to be present in both the cytoplasm and the nucleus (see higher magnification insets, Figure 2 A and B). In breast tissue, once again, AR and ART-27 are both expressed in nuclei of epithelial cells. However, AR appears to be more abundant in the distal regions of the gland (observed as the smaller round gland indicated by the arrow in Figure 2 E) than in the more proximal part of the gland (the large central gland), while ART-27 protein appears to be abundant in both regions. Neither protein is ubiquitously expressed in epithelial cells of all tissue types, however, because there is little positive immunoreactivity in epithelial cells of the colon (not shown). As a control for antibody specificity, tissues were incubated in the presence of the primary antibody plus the immunizing peptide; these tissues show no immunoreactivity indicating that all of the observable staining is due to antibody recognition of the ART-27 C-terminal peptide. Additionally, incubation of the sections with secondary antibody only (Figure 2 C and F) indicate that the secondary antibody does not contribute to the observed staining pattern. Therefore, the pattern of ART-27 protein expression is epithelial cell specific.

Closer examination of immunoreactivity with the ART-27 antibody in human prostate tissue sections shows strong epithelial cell staining and little to none in the stroma of adult human prostate tissue (Figure 3A). To further verify the cell type specificity of ART-27 protein expression, Western blot analysis was performed using lysates from explant cultures of primary human epithelial and stromal cells (26). Consistent with the immunohistochemistry results, ART-27 is highly expressed in epithelial cells and expressed at low levels, if at all, in stromal cells (Figure 3B).

ART-27 is expressed in rat prostate epithelial cells undergoing androgen-dependent proliferation

Since studies in human prostate tissue (above) indicated that ART-27 and AR are both expressed in prostate epithelial cells, a rat model of androgen depletion/reconstitution was used to examine the effects of androgen-mediated prostate growth and differentiation on ART-27 protein expression (27). Similar to the expression of ART-27 in human prostate tissue, immunohistochemistry performed on rat prostate tissue samples indicated that ART-27 protein is expressed in prostate luminal epithelial cells, but not in stromal cells (Figure 4A). To androgen

deprive the animals, rats were castrated to cause withdrawal of testicular androgens and regression of the prostate gland. Androgens were then re-administered for 24 and 48 hours, resulting in cellular proliferation and growth of the prostate. Prostate glands were dissected from the animals at the indicated time points and lysates were prepared from each gland and used for Western blot analysis. The filters were incubated with antibodies against ART-27 as well as proliferating cell nuclear antigen (PCNA, a marker for cellular proliferation), clusterin (a protein that increases upon prostate involution and apoptosis (28-30)), and MAPK (used as an internal loading control). Following castration, PCNA expression is abolished and clusterin expression is greatly increased, supporting the induction of apoptosis in the prostate epithelium (Figure 4B, cas). Upon administration of exogenous androgens, a marked increase in PCNA is noted with rapid reduction of clusterin expression, thereby confirming the mitotic role of androgens in this model (A24 and A48). ART-27 protein is dramatically reduced following androgen withdrawal (cas), but is abundant when androgens are available (con, A24 and A48). Although it is not evident in this figure, longer exposure of the film from the blot incubated with ART-27 antibody does detect ART-27 in the samples from the castrated animals (cas). Therefore, in this model, the expression of ART-27 is associated with an abundance of differentiated prostate epithelial cells and is reduced along with diminishing numbers of epithelial cells under conditions of androgen deprivation. Subsequent experiments are designed to determine if ART-27 may play a role in androgen-dependent prostate growth or differentiation.

Regulated expression of ART-27 in the androgen sensitive LNCaP cell line causes decreased cell proliferation

To determine the effects of ART-27 on prostate cancer cell growth we constructed stable cell lines that express an epitope tagged variant of ART-27 under the regulation of a tetracycline promoter in androgen-sensitive LNCaP prostate cancer cells. Parental LNCaP cells were transfected with the pTet-On vector and resulting clones were screened for Tet-dependent activation by measuring pRevTRE-luciferase reporter gene activity. A LNCaP clone displaying tight Tet-dependent activation was then transfected with pRevTRE:ART-27 (C-FLAG) prior to selection of resistant colonies. Figure 5A shows expression of endogenous (ART-27) and ART-27 (C-FLAG) mRNA in one of the resulting clones in the presence and absence of R1881 and the tetracycline analog, doxycycline, as examined by Northern blot analysis. As expected, ART-27 (C-FLAG) rev/tet mRNA is upregulated in the presence of doxycycline (Figure 5A, lanes 2 and 4). However, neither endogenous ART-27 RNA levels (lower arrow) or ART-27 (C-FLAG) rev/tet RNA (upper arrow) levels are changed in response to the presence or absence of androgens (compare lanes 1 and 2 versus lanes 3 and 4) indicating that androgens do not regulate the transcription of ART-27.

Since subsequent experiments are designed to look at the effect of exogenous ART-27 on androgen mediated cell growth in LNCaP cells it was first determined if endogenous ART-27 protein levels are regulated by androgens, as might be suggested by the depletion of ART-27 protein levels in the castrated rat prostate cell lysates (Figure 4B). For this purpose, ART-27 protein levels were determined in parental LNCaP cells in the presence and absence of R1881 (Figure 5B). Western blot analysis of lysates made from androgen starved or androgen stimulated LNCaP prostate cancer cells is shown in Figure 5B. The abundance of ART-27 protein in androgen starved cells (Figure 5B, lane 1) is identical to the levels of ART-27 in androgen treated LNCaP cells (Figure 5B, lane 2) suggesting that androgens do not affect the steady state levels of ART-27 protein.

Analysis of ART-27-FLAG protein expression in response to doxycycline is shown in Figure 6A. As expected, treatment of an ART-27-FLAG clone #1 with increasing concentrations of doxycycline results in higher levels of ART-27 protein expression. As a preliminary step to the thymidine uptake experiments described below, it was also determined that endogenous levels of AR expression are not affected by treatment of the cell clones with doxycycline (Figure 6B).

To determine the effect of ART-27 on androgen mediated LNCaP cell proliferation, DNA synthesis was determined by incorporation of ^{3}H-thymidine into cellular DNA. Figure 6 (C and D) shows analysis of ART-27-FLAG clone #1 (C) and clone #2 (D) in the presence and absence of doxycycline and R1881. Both clones demonstrate regulation of ART-27-FLAG in response to doxycycline. Figure 6C shows a representative experiment in which ^{3}H-thymidine was added to the cells 1 hour prior to sample analysis. As expected, there is very little incorporation of thymidine into DNA in the absence of androgens whether the cells are treated with doxycycline or not (Figure 6C, 0nM conditions). In the presence of R1881 (Figure 6C, 0.05nM) thymidine is incorporated into the cellular DNA as expected. However cells expressing ART-27 FLAG (0.05nM + Dox), show diminished thymidine incorporation compared to those that do not (0.05nM -Dox) at the 48h and 72h treatment with R1881. Analysis of an additional clone (ART-27-FLAG clone #2) shows a similar trend (Figure 6D). Again the cells show thymidine incorporation in response to androgens (compare 0nM –Dox to 0.05nM -Dox). In this clone, expression of ART-27-FLAG in the presence of 0.05nM R1881 (0.05nM +Dox) also reduces thymidine uptake compared to the cells grown in the absence of ART-27-FLAG (0.05nM –Dox). In fact, in clone #2 the presence of ART-27-FLAG thymidine uptake is reduced to levels seen in the R1881 minus conditions (compare 0.05nM +Dox to 0nM +Dox). As a control it was shown that doxycycline alone at the concentration utilized does not inhibit parental LNCaP cell thymidine uptake (not shown).

The decreased ability of ART-27-FLAG LNCaP cell clones to synthesize DNA in the presence of doxycycline induced ART-27 gene expression is corroborated by the fact that we were not able to make stable LNCaP cell lines that over-expressed ART-27 constitutively. In these experiments ART-27 was sub-cloned into the pIRES2 vector (Clontech) that utilizes an internal ribosome entry site (IRES) to express the protein of interest (ART-27) on the same transcript as a selectable marker (neomycin). While hundreds of LNCaP colonies grew upon selection when transfected with pIRES2 alone, no colonies were evident on plates transfected with ART-27-pIRES2, again suggesting that increased levels of ART-27 protein inhibit or prevent LNCaP cell growth.

ART-27 expression is decreased in prostate cancer

As the above results suggested that ART-27 inhibits androgen-mediated prostate cell growth, we evaluated the expression of ART-27 protein in benign (non-cancerous) and malignant (cancerous) human prostate tissue through immunohistochemical staining of paraffin-embedded radical prostatectomy (n=22) specimens. Consecutive tissue sections stained with affinity purified ART-27 antibody or with an AR antibody show nuclear staining of normal prostate glandular epithelium (Figure 7 A and B) as previously noted. Additionally, insets in panels A and B show that neither ART-27 nor AR exhibit immunoreactivity in prostate basal cells (see vertical arrows). Immunohistochemical staining of sections containing regions of adenocarcinoma (glandular epithelial cancer) indicate that ART-27 staining in prostate cancer is decreased (indicated by the black box in Figures 7 C and E) relative to that observed in adjacent

benign prostatic glands (indicated by arrows in Figure 7 C and E). AR staining of a consecutive section shows uniform staining in benign and malignant glands thereby providing a positive control for the integrity of the tissue specimen (Figure 7 D and F). A higher magnification comparison of AR and ART-27 expression in an additional prostate cancer tissue specimen again confirms robust levels of AR and negligible levels of ART-27 (Figure 8 A and B).

Decreased ART-27 staining was observed in all 22 cancer specimens examined. Decreased ART-27 signal could not be attributed to tissue damage (caused by over-fixation, under-fixation, cauterization, etc.) since only slides that showed positive immunoreactivity with anti-AR antibody were included in the study. The majority of samples tested were moderately differentiated (Gleason score 5-7), with a minority representing poorly differentiated disease (Gleason 8-10). In comparing the few samples tested, however, staining patterns of the malignant glands appear similar regardless of the degree of differentiation.

In evaluating sections containing high grade prostatic intraepithelial neoplasia (PIN), a condition thought to be a precursor to prostate cancer, strong ART-27 staining was noted within the dysplastic (abnormal looking, but non-cancerous) epithelium (Figure 8 C and D). Thus, reduction in ART-27 expression appears to be a relatively late event in prostate carcinogenesis. Based collectively upon immunohistochemical staining patterns in prostate cancer and effect on prostate cancer cell growth, we speculate that ART-27 may play a role in promoting androgen-dependent epithelial differentiation thereby reducing tumor progression or proliferation rate. Its potential role in carcinogenesis remains to be defined.

ART-27 in human fetal development

The disappearance of ART-27 protein in prostate cancer progression and consequent de-differentiation suggests that ART-27 may play a role in luminal epithelial cell differentiation. To examine ART-27 protein expression during developmental differentiation, we assessed the pattern of ART-27 protein in the region of the urogenital sinus from which the prostate develops in human fetal tissue. Sections through the urogenital sinus region of a 15 week old fetus show a cell free region in the center which is the lumen of the urethra (indicated by the U in Figure 9 A and B) adjacent to columns of epithelial cells that are surrounded by mesenchymal or stromal tissue (str). Epithelial outgrowths from the urethra grow and branch into the surrounding mesenchyme to form the prostate buds (PB) or tubules. Incubation of a tissue section with ART-27 antibody shows that ART-27 protein is found in the layer(s) of the epithelial cells adjacent to the lumen of the urethra, but not in stromal cells or in the prostatic buds at 15 weeks of development (Figure 9A). Incubation of a tissue section with anti-AR antibody, on the other hand, shows staining of the epithelial cells surrounding the lumen, as well as staining in stromal cells, and epithelial cells in the central region of the prostatic buds (Figure 9B). Tissue from the urogenital region of a 21 week old fetus was also stained with ART-27 antibody. Since the urethra is much larger at this point in development only a portion of the urethra is visible in the photograph (Figure 9C). Again ART-27 is present in the luminal cells adjacent to the lumen of the urethra. A higher magnification photograph (Figure 9D, top) indicates that the single cell layer of columnar shaped cells adjacent to the lumen stain strongly. This is in contrast to the pattern of AR protein expression. Incubation of a consecutive section with anti-AR antibody shows that AR is strongly expressed in stromal cells as well as in multiple layers of epithelial cells adjacent to the lumen of the urethra (Figure 9D, bottom). The arrowheads indicate identical cells that are positive for the presence of both ART-27 and AR. These results suggest that in the urogenital sinus ART-27 is present in differentiated cells with an apical surface

adjacent to the lumen rather than in proliferative cells closer to the basal cell layer. This idea is supported by the fact that in tissue from the urogenital region of a 15 week old fetus (Figure 9A) there is little expression of ART-27 in the prostatic buds. At 21 weeks however, there is expression in some prostatic buds that have begun to form lumen (see Figure 9 C, arrowheads). By 24 weeks most of the prostatic buds have developed a lumen and nuclear expression of ART-27 is now observable in most of the prostatic buds (not shown). Thus, expression of ART-27 tightly correlates with differentiation of prostate luminal epithelial cells in human fetal development.

Discussion

Attempts to understand the role of AR in prostate cell growth modulation or differentiation suggest that its effects are not simple, and that cellular context will play an important role (31) (32) (33,34). Androgen receptor coactivators such as ART-27, are likely to modulate AR activity and determine differentiation and proliferation programs of prostate cell gene transcription.

Concise transcriptional regulation is a prerequisite for the development of highly specialized cell types that comprise multi-cellular organisms. One mechanism underlying such specificity is the restricted expression pattern of components of the transcriptional machinery to specialized cell types. For example, $TAF_{II}105$, a component of the transcription factor TFIID, is restricted to the granulosa cells of the ovary follicle and regulates a subset of genes necessary for normal ovarian follicular development (35). The strikingly cell type specific pattern of ART-27 protein localization during fetal urogenital development suggests that ART-27 regulates a subset of AR responsive genes in specialized cell types. For example, while AR is expressed in multiple cell layers of the urogenital sinus, ART-27 is expressed in a single cell layer adjacent to the lumen. In prostate, ART-27 is not present in prostatic buds at day 15, but becomes detectable at later stages that correlate with the differentiation of luminal epithelial cells and the appearance of a lumen. As the majority of proteins expressed by the differentiated prostate epithelium are secretory in nature, the presence of a lumen indicates cellular differentiation. Thus, ART-27 protein expression strongly correlates with prostate cell differentiation during organ development.

Within the mature prostate, ART-27 expression appears to remain unique to differentiated epithelial cells. Little to no expression is observed within the proliferative (basal cell) compartment of the glandular epithelium or the stroma. Within the luminal epithelium, variable intensity of ART-27 nuclear staining is often observed within cells uniformly staining for AR. This suggests a functional role for ART-27, perhaps dependent upon cell cycle, stage of cellular maturation, or secretory function.

As ART-27 is present in both benign and pre-malignant (HGPIN) epithelium, but relatively absent in prostate cancer, the loss of ART-27 expression appears to be a relatively late event in carcinognesis. Reintroduction of ART-27 into a prostate cancer cell line by stable transfection results in markedly decreased androgen-stimulated proliferation. Further, complete cessation of growth appears to occur upon attempts to constitutively express ART-27 within the cell line. These data are consistent with the hypothesis that ART-27 functions in initiation or maintenance of an AR mediated program of prostate cell differentiation.

Androgen receptor coactivators and corepressors are likely to play a role in androgen dependent and androgen-independent prostate cancer. The most effective means of treating prostate cancer is by androgen ablation therapy, which causes prostate tissue along with prostate

tumors, to regress. While cutting off the supply of androgens is beneficial in the short term, some prostate cancer cells inevitably begin to grow in a hormone independent fashion, and at this point the disease is poorly treated. Multiple mechanisms might explain this hormone refractory disease including androgen receptor mutation and/or upregulation and altered recruitment of AR coactivators or corepressors to the AR (36) (37). The idea that steroid receptor coactivators might be instrumental in cancer progression has been realized with the discovery that, Tamoxifen, the estrogen antagonist widely used to treat breast cancer, acts by recruiting the nuclear receptor corepressor, NcoR and preventing co-activator binding to ER (38) (39,40). A recent report indicates that recurrent prostate cancers express higher than normal levels of the steroid hormone coactivators, transcriptional intermediary factor 2 (TIF2), and SRC-1 coactivator (Steroid Receptor Coactivator-1) genes (41). In addition, *in situ* analysis of multiple androgen receptor coactivator expression levels showed that mRNA expression of the coactivators varied between normal and malignant prostate tissue samples (42). Effects on normal prostate cell growth have also been demonstrated in animal studies by targeted mutagenesis of the SRC-1 gene (43). However, these effects are relatively minor and therefore suggest that compensatory mechanisms exist and/or that additional co-regulators are required for androgen mediated cell growth.

Although the AR N-terminus has been shown to be critical for AR transactivation, the expression of AF-1 binding proteins has not been evaluated during prostate cancer progression. Our data indicate that ART-27 expression is altered during prostate cancer progression, and therefore may play an important role in this disease. It is currently unknown how ART-27 is regulated in normal prostate cells or in prostate cancer. The cell type specificity of ART-27 expression suggests that it is not directly regulated by androgens since ART-27 is expressed in a far more restricted pattern than AR. In addition, androgen treatment does not induce ART-27 mRNA or protein in LNCaP cells and there are no decipherable androgen response elements in the promoter of ART-27 (unpublished results, Li and Garabedian). However, it is likely that ART-27 protein expression is indirectly regulated by localized secretion of cytokines or growth factors in response to androgen signaling.

In conclusion, a growing body of evidence suggests that co-regulators of steroid hormone receptors impart functional flexibility, thus allowing the receptors to respond differentially contingent upon their biological context (44). Our results are consistent with the idea that the androgen receptor coactivator ART-27, is a cell type restricted cofactor, and promotes androgen dependent prostate epithelial cell differentiation, thereby inhibiting cellular proliferation.

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Figure Legends

Figure 1 Characterization of ART-27 antibody. A) Shows that anti-C-terminal ART-27 antibody recognizes HA-tagged ART-27 (H), FLAG-tagged ART-27 (F) and endogenous ART-27 (E) (top panel) and that these molecules are similarly recognized by an antibody made against full length ART-27 protein fused to glutathione S-transferase (gst) (bottom panel). Samples shown on the Western blot are from 293 cells transfected with HA-tagged ART-27 (lanes 2 and 6), FLAG-tagged ART-27 (lanes 1 and 5), or with vector alone (lanes 3,4, and 7). Samples are either total cell lysates (lanes 1-3) or immunoprecipitated with the indicated antibodies (lanes 4-7). Figure 1 A bottom panel shows that antibody made against a full length ART-27 gst fusion protein recognizes the same immunoprecipitated ART-27 variants as the anti-ART-27 C-terminal antibody. Lysates used in lanes 5,6, and 7 are identical in the top and bottom panels; the blots were incubated with the indicated antibodies. B) Shows that over-expressed untagged ART-27 is the same size as endogenous ART-27. 293 cells were transfected with untagged ART-27 (lane 1) or vector alone (lane 2) and lysates were immunoprecipitated with anti-ART-27 antiserum prior to blotting with affinity purified anti-ART-27 (C-terminus).

Figure 2 Expression of ART-27 and AR in normal human breast and prostate tissues. Immunohistochemistry done on tissues incubated with anti- ART-27 antibody (A and D), anti-AR antibody (B and E) and secondary antibody only (C and F) in prostate and breast tissue shown at 400x magnification. To clarify cell type specific staining and subcellular staining, insets show the same sample at 1000x magnification. Positive reactivity with the antibodies is visualized as brown staining. Prostate tissue and breast tissue show strong nuclear staining for both AR and ART-27. No staining is evident in the presence of the secondary antibody alone, or when anti-ART-27 antibody is incubated with the immunizing peptide (not shown).

Figure 3 ART-27 expression in the prostate is restricted to luminal epithelial cells. A) Shows immunohistochemical staining of human glandular prostate tissue with ART-27. The epithelial cell layers at the top and bottom of the tissue (indicated by arrowheads) show nuclear staining of ART-27, while the centermost horizontally oriented stromal cells (Str) do not stain. Magnification is 400x. B) Shows Western blot analysis utilizing lysates made from primary prostate stromal or primary prostate epithelial cells and analyzed with anti-ART-27 antibody.

Figure 4 ART-27 expression in a rat model of androgen dependent prostate cell growth and differentiation. A) Shows immunohistochemical staining of normal adult rat prostate tissue with affinity purified anti-ART-27 antibody. Epithelial cells (indicated by arrows) surrounding the lumen (L) of the large glands are strongly positive for nuclear ART-27 staining. B) Shows regulation of proteins under conditions of androgen depletion/reconstitution. Lysates were made from rat prostates under the following conditions: untreated (con), 96 hours post-castration (cas), 96 hours post-castration plus 24 hours treatment with androgens (A24), and 96 hours post-castration plus 48 hours treatment with androgens (A48). The lysates were then normalized for protein concentration and used for Western blot analysis with ART-27 antibody, or MAP kinase (MAPK) antibody as an internal control for protein loading of the gel. The Western blots were also probed with PCNA and clusterin antibodies as indicated.

Figure 5 Steady state ART-27 mRNA and protein levels are not androgen regulated. Panel A is a Western blot showing lysates from LNCaP cells not treated (lane 1) or treated with 1 nM R1881 for 48 hours (lane 2) and blotted with anti-ART-27 antibody. Lane 3 is a control showing over-expressed untagged ART-27. Panel B) shows a northern blot hybridized with an ART-27 cDNA probe. Lanes show RNA made from the doxycycline (dox) inducible ART-27-FLAG clone #1 treated with or without Dox and R1881 as indicated for 48 hours. The ART-27 rev/tet signal is generated from the doxycycline-inducible ART-27 construct. While ART-27 rev/tet transcripts are evident only in the presence of dox (ART-27 rev/tet), endogenous levels of ART-27 (bottom arrow) do not change in the presence of R1881 or dox. Transcripts from the tet-inducible ART-27 vector (rev/tet) are larger than the endogenous ART-27 gene as the ART-27 cDNA does not include a poly A sequence and the transcript is extended into the adjacent LTR within the vector.

Figure 6 Inhibition of androgen-mediated LNCaP cell growth by regulated ART-27 expression. A) Shows Western blot analysis of lysates from doxycycline-inducible ART-27-FLAG cell clone #1 under conditions of increasing doxycycline. The blot was incubated with anti-FLAG antibody. B) Shows that doxycycline does not affect steady state levels of endogenous AR. Lysates are made from doxycycline inducible ART-27FLAG clone #1 treated as indicated for 72 hours and blotted with anti-AR antibodies. C) Shows ^{3}H-thymidine incorporation into cellular DNA at 48 and 72 hours in ART-27-FLAG clone #1 in the presence (0.05nM) or absence (0nM) of R1881 and in the presence (+Dox) or absence (-Dox) of 1μg/ml doxycycline. Each time point was done in triplicate and the graph shown is representative of multiple experiments. D) Shows thymidine incorporation into cellular DNA at 48 and 72 hours in ART-27-FLAG clone #2 in the presence (0.05nM) or absence (0nM) of R1881 and in the presence (+Dox) or absence (-Dox) of 1μg/ml doxycycline.

Figure 7 Lack of ART-27 expression in prostate cancer. Normal prostate (A and B) or cancer containing prostate (C-F) were stained with AR antibody (right panels) or ART-27 antibody (left panels) as indicated. Cancer samples from three different individuals are shown in Figure 7 (C-F) and Figure 8 (A and B). In each case consecutive samples are treated with either ART-27 antibody or AR antibody. As expected, both AR and ART-27 are predominantly expressed in the nuclei of normal prostate epithelium (A and B). The insets show the same samples at 1000x magnification. Neither AR nor ART-27 exhibit staining in basal cells (vertical arrows A and B, insets). The horizontal arrow (B, inset) indicates an AR positive staining stromal cell. A tissue region typical of prostate cancer containing small irregularly shaped glands is demarcated by the black box in panels C-F. While AR is present in the cancerous regions (D and F), ART-27 is not expressed in the cancerous glands (C and E). Both ART-27 and AR are expressed, however, in adjacent non-cancerous glands that are either normal or benign hyperplastic glands (indicated by arrows in C, E and F). Images are shown at 200x magnification.

Figure 8 ART-27 expression in cancer versus in high grade PIN. Shows consecutive sections of prostate cancer tissue stained with either anti-ART-27 antibody (left panels) or with anti-AR (right panels). Samples are from different individuals than shown in Figure 7. As above, adjacent photographs are from consecutive sections of the same tissue sample. Panels A and B

show expression of AR and ART-27 in prostate cancer. Panels C and D show expression of AR and ART-27 in high grade PIN. Images are shown at 400x magnification.

Figure 9 ART-27 and AR expression in human fetal prostate development. Shows paraffin sections of 15 week old and 21 week old human fetal urogenital sinus. Prostatic buds (PB) are indicated by arrows in A-C and the lumen of the urethra is always indicated for orientation (U). Tissue samples are incubated with either anti-ART-27 antibody (A, C and D, top) or anti-AR antibody (B and D, bottom). Panel A shows that at 15 weeks ART-27 is expressed in epithelial cells adjacent to the lumen of the urethra (a small patch of epithelial cells is indicated by the box), but not in the prostate buds (PB) or surrounding stroma (Str). At 21 weeks (Panel C), the presence of ART-27 is also detected in epithelial cells adjacent to the lumen of the urethra, and is not detected in the surrounding stroma (Str). However, ART-27 is now also detectable in some of the larger prostatic buds (PB) that have formed a lumen (positive ART-27 reactivity in the buds is indicated by arrows). Panel B shows that AR is expressed in epithelial cells adjacent to the lumen of the urethra as well as in the stromal cells surrounding the glands and in the prostate buds. A higher magnification photograph of the epithelial cell layer between the lumen of the urethra (U) and stromal cells (Str) is shown in D. Staining of the two consecutive tissue sections with antibody against ART-27 (D, top) and AR (D, bottom) shows that ART-27 primarily stains the nuclei of the single layer of epithelial cells adjacent to the lumen while AR appears to stain multiple epithelial cell layers and stromal cells. Arrows are used to indicate three cells that stain positively for both ART-27 (D, top) and AR (D, bottom).

Figure 1

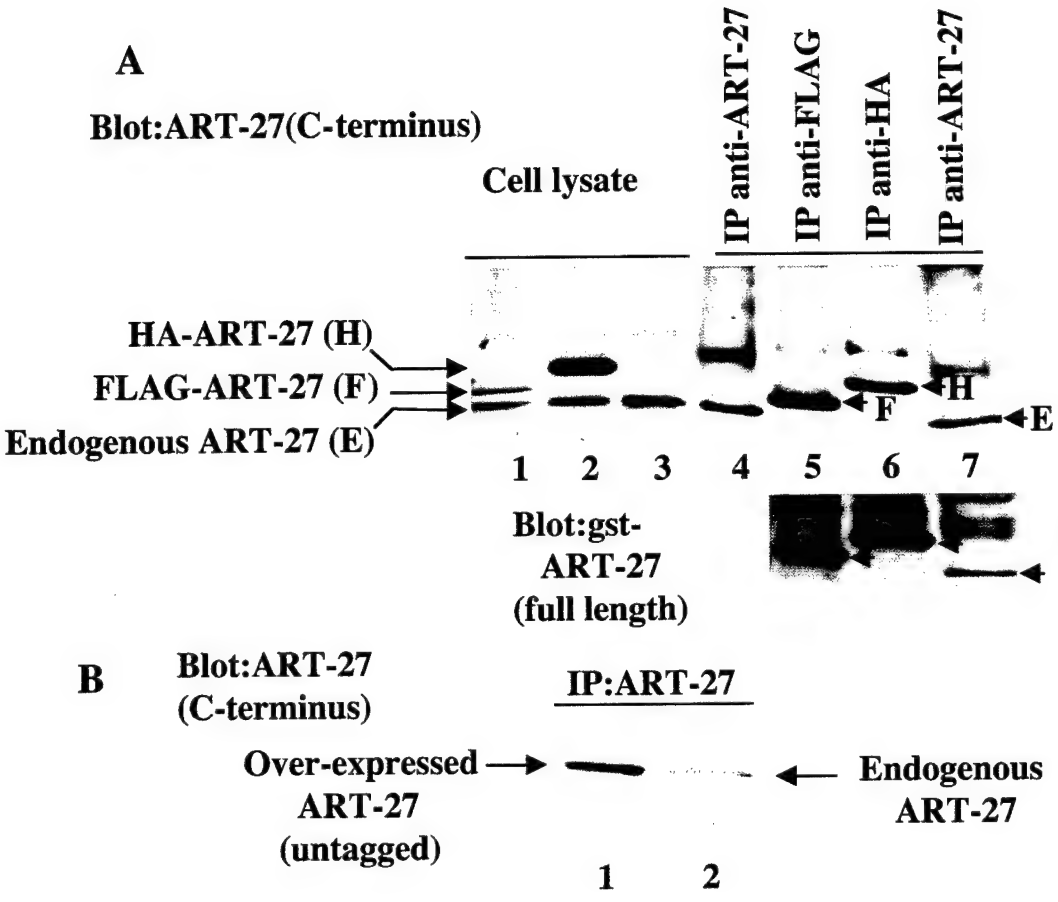
A
Blot:ART-27(C-terminus)
Cell lysate
IP anti-ART-27
IP anti-FLAG
IP anti-HA
IP anti-ART-27
HA-ART-27 (H)
FLAG-ART-27 (F)
Endogenous ART-27 (E)
F
H
E
1 2 3 4 5 6 7
Blot:gst-ART-27 (full length)
B
Blot:ART-27 (C-terminus)
IP:ART-27
Over-expressed ART-27 (untagged)
Endogenous ART-27
1 2

Figure 2

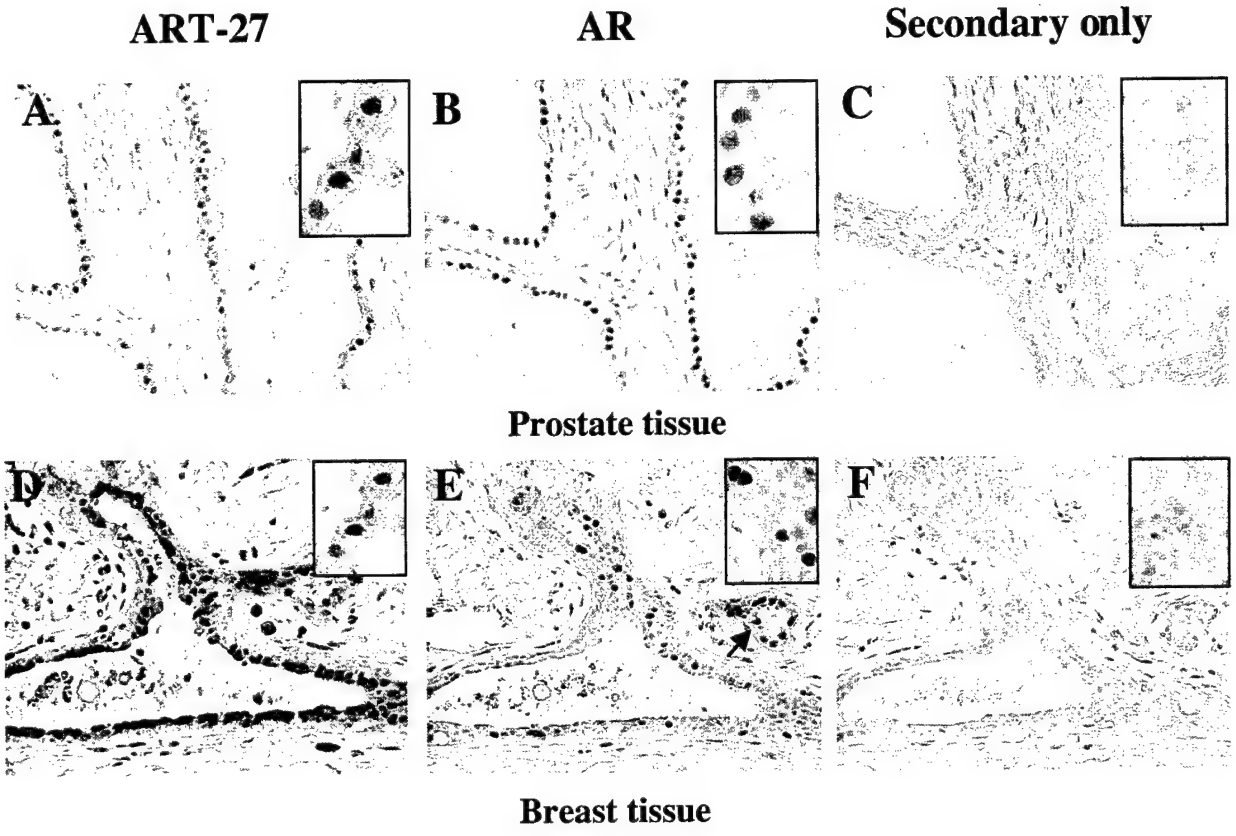
ART-27
AR
Secondary only
A
B
C
Prostate tissue
D
E
F
Breast tissue

Figure 3

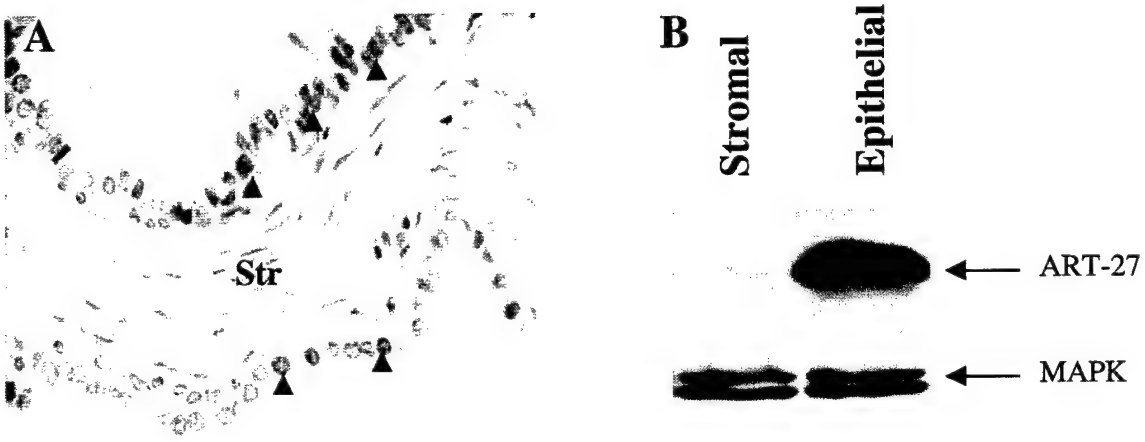

Figure 4

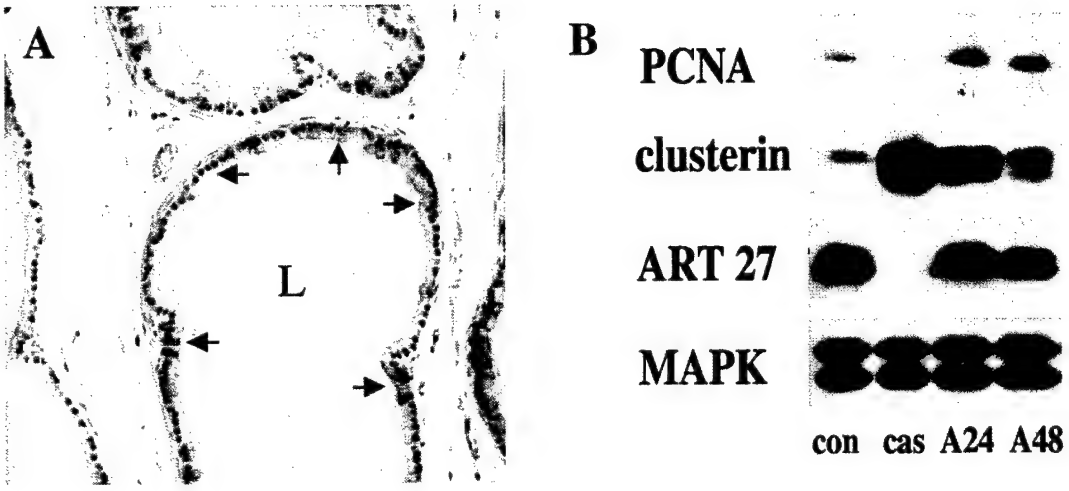

Figure 5

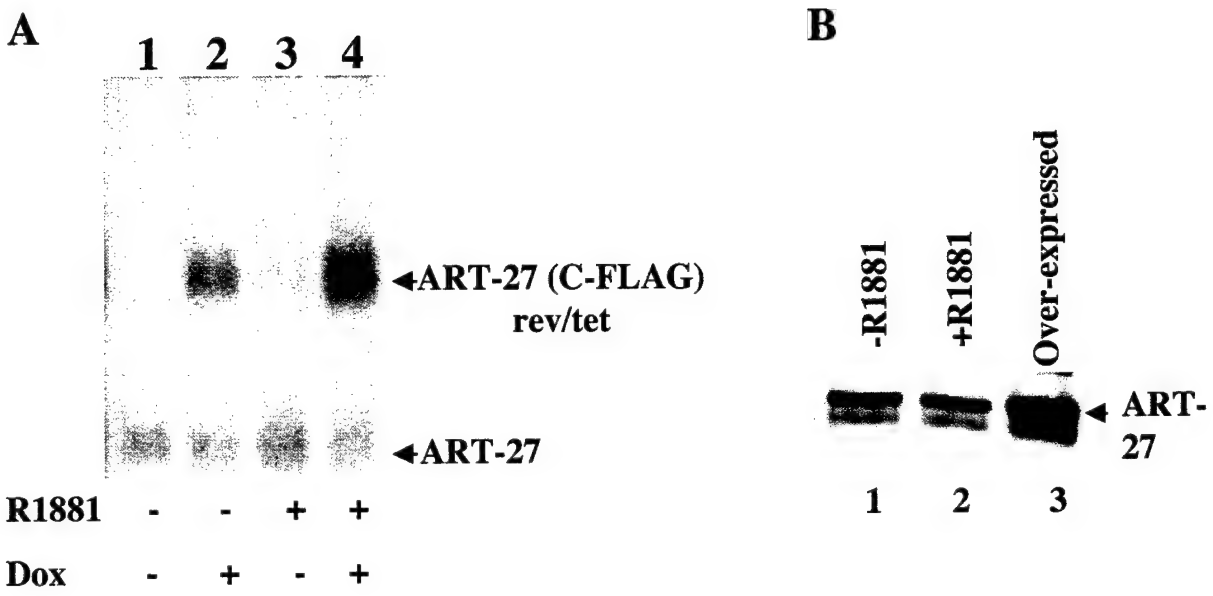
A
1 2 3 4
ART-27 (C-FLAG)
rev/tet
ART-27
R1881 - - + +
Dox - + - +
B
-R1881
+R1881
Over-expressed
ART-27
1 2 3

Figure 6

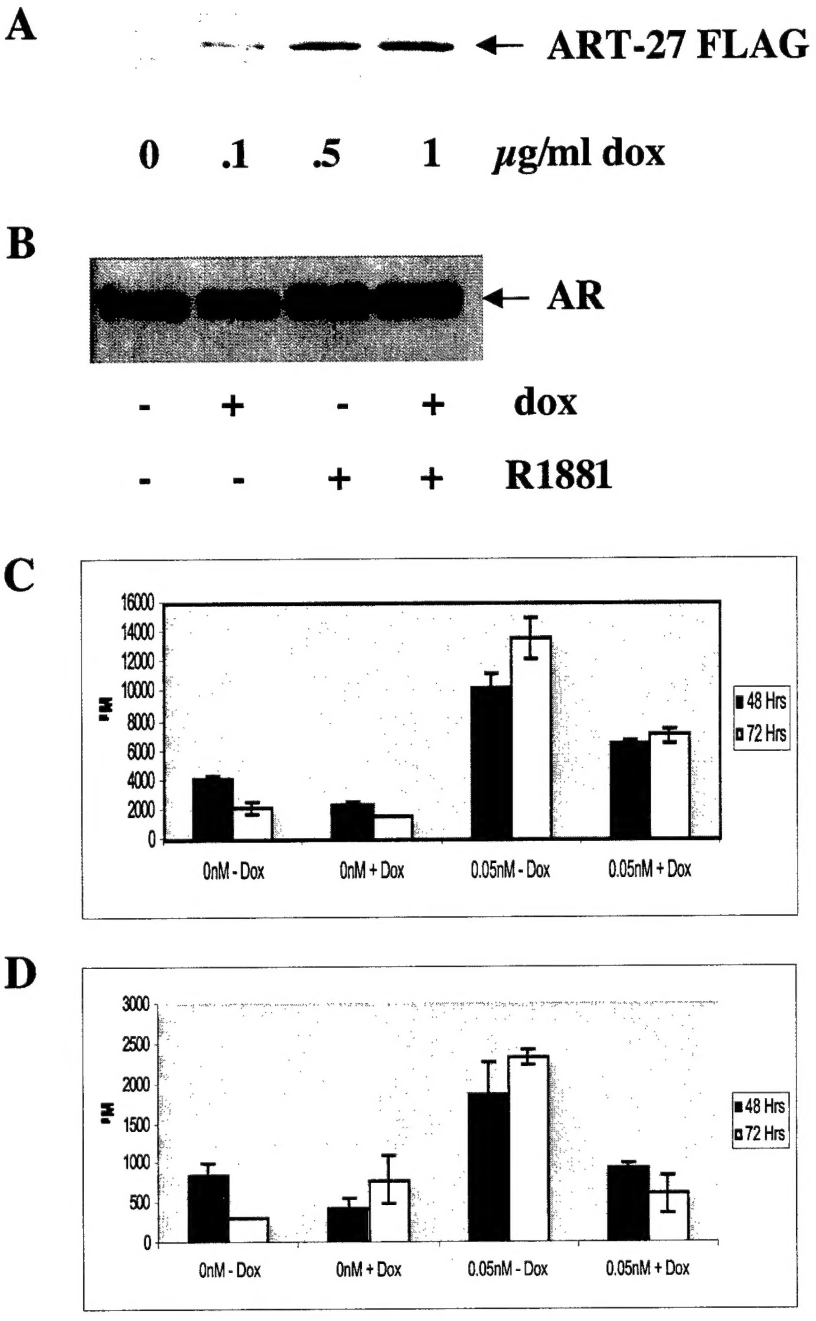
A
ART-27 FLAG
0 .1 .5 1 μg/ml dox
B
AR
- + - + dox
- - + + R1881
C
16000
14000
12000
10000
8000
6000
4000
2000
0
0nM - Dox
0nM + Dox
0.05nM - Dox
0.05nM + Dox
48 Hrs
72 Hrs
D
3000
2500
2000
1500
1000
500
0
0nM - Dox
0nM + Dox
0.05nM - Dox
0.05nM + Dox
48 Hrs
72 Hrs

Figure 7

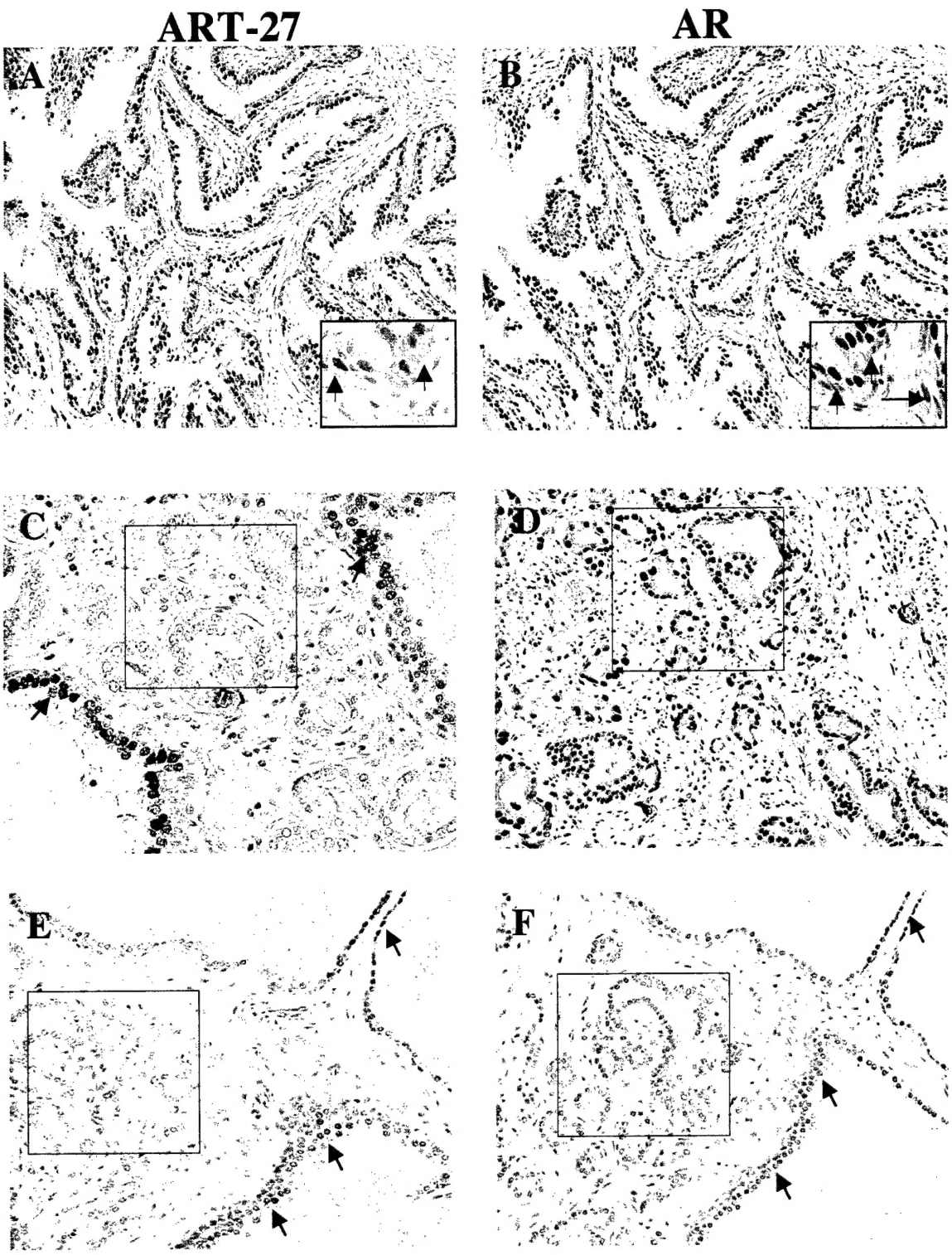

Figure 8

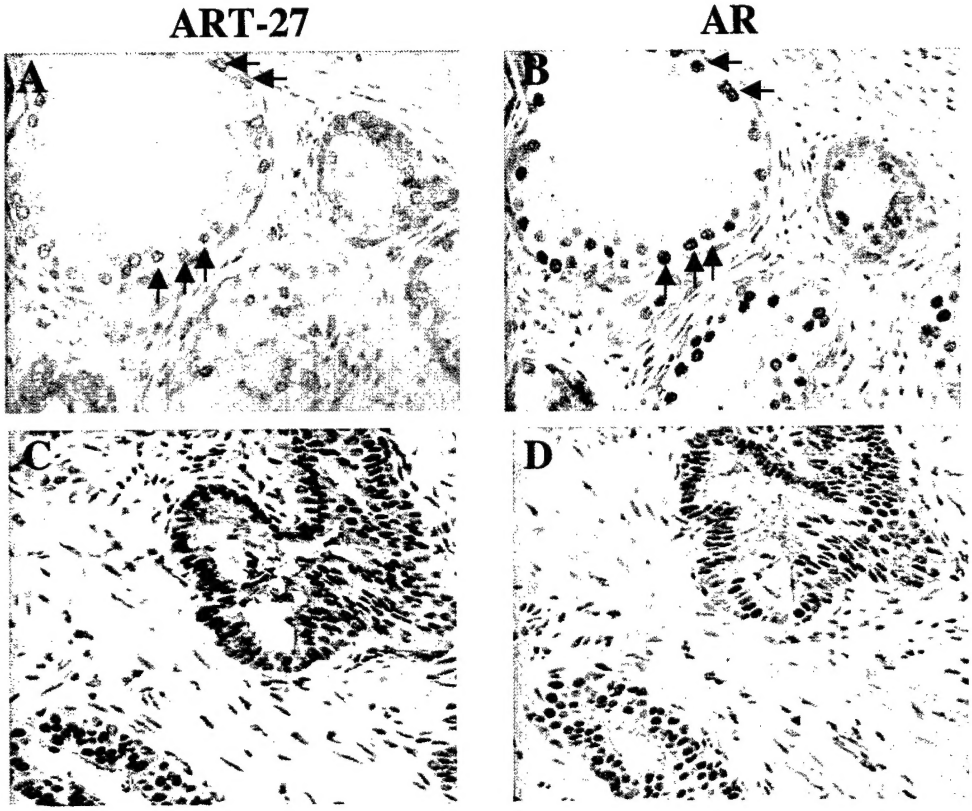

Figure 9